Study of the effects of chlorogenic acid supplementation during in vitro maturation on the developmental competence of porcine oocytes

ブタ卵母細胞の発生能力に及ぼす体外成熟中のクロロゲン酸添加の影響に関する研究

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ABBREVIATION

ANOVA	analysis of variance
BSA	Bovine serum albumin
CGA	Chlorogenic acid
COCs	Cumulus-oocyte complexes
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphat-buffered saline
eCG	Equine chorionic gonadotropin
EGF	Epidermal growth hormone
FBS	Fetal bovine serum
hCG	Human chorionic gonadotropin
HEPES	Hydroxyethyl-piperazineethane-sulfonic acid buffer
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
DPBS	Dulbecco’s modified phosphate-buffered saline
m-PBS	Modified phosphate-buffered saline
PBM	Porcine blastocyst medium
PFM	Porcine fertilization medium
POM	porcine oocyte medium
PZM-3	pig zygote medium 3
PZM-5	pig zygote medium 5
PLSD	Protected least significant difference
ROS	Reactive oxygen species
SCNT	Somatic cell nuclear transfer
TCM 199	tissue culture medium-199
TUNEL	terminal deoxynucleotidyl transferase nick-end labelling

SUMMARY

Improving the quality of in vitro derived porcine embryos and maintaining their in vivo development after transfer is crucial in both scientific and commercial points of view. Oxidative stress is a certain threat to porcine oocytes and in vitro derived embryos since these cells are removed from their natural habitats, leading to the lack of maternal antioxidant factors. The present studies were conducted to investigate antioxidant effects, anti-heat stress effects of chlorogenic acid (CGA) during in vitro maturation (IVM) on the developmental competence of porcine in vitro derived embryos with/without electro-stimulation treatment.

The first study was aimed to investigate the effects of CGA supplementation during IVM on in vitro development of porcine oocytes in order to improve the porcine in vitro production (IVP) system. Oocytes were matured either without (control) or with CGA (10, 50, 100 and 200 μM). Subsequently, the matured oocytes were fertilized with capacitated sperm, and the presumptive zygotes were subsequently cultured in vitro for 7 days. The rates of maturation, fertilization and blastocyst formation of oocytes matured with 50 μM CGA were significantly ($p < 0.05$) higher than those of the control oocytes. Hydrogen peroxide (H_2O_2) is one of the reactive oxygen species, and this chemical induces DNA damage to porcine oocytes. When oocytes were matured in IVM medium added with 1 mM H_2O_2 to assess the protective effect of CGA, 50 μM CGA supplementation improved the maturation rate and the proportion of DNA-

fragmented nuclei in oocytes compared with control oocytes matured without CGA. Moreover, when oocytes were matured with either 50 μM CGA (control) or caffeic acid (10, 50 and 100 μM), the rates of maturation, fertilization and the blastocyst formation of oocytes matured with 50 μM CGA were similar to those of oocytes matured with 10 and 50 μM caffeic acid. Our results suggest that CGA has comparable effects to caffeic acid, and IVM with 50 μM CGA is particularly beneficial to IVP of porcine embryos and protects oocytes from DNA damage induced by oxidative stress. Therefore, supplementation of CGA to the maturation medium has a potential to improve porcine IVP system.

In the second study, we investigated the effects of CGA on porcine oocyte maturation under heat stress and subsequent embryonic development after parthenogenetic activation. For IVM at 41.0°C (hyperthermic condition), supplementation of the maturation medium with 50 μM CGA significantly improved the percentage of matured oocytes and reduced the rate of apoptosis relative to oocytes matured without CGA ($p < 0.05$). CGA treatment of oocytes during IVM under hyperthermia tended to increase ($p < 0.1$) percentage of blastocyst formation after parthenogenesis and significantly increased ($p < 0.05$) the total cell number per blastocyst relative to oocytes matured without CGA. For IVM at 38.5°C (isothermic condition), CGA significantly improved the rate of blastocyst development compared with oocytes matured without CGA ($p < 0.05$), but did not affect oocyte maturation, apoptosis rate or the number of cells per embryo. Omission of all antioxidants from the IVM medium significantly

reduced the rate of oocyte maturation, but the rate was restored upon addition of CGA. These results demonstrate that CGA is a potent antioxidant that protects porcine oocytes from the negative effects of heat stress, thus reducing the frequency of apoptosis and improving the quality of embryos.

Electroporation is a method of choice to introduce an exogenous gene into embryos for transgenic animal production. Although this method is considered practical and effective, embryonic damage caused by electro-stimulation treatment remains a major problem. Thus, another study was conducted to evaluate the optimal culture system for electro-stimulation treated porcine embryos by supplementation of chlorogenic acid (CGA) during IVM. The oocytes were treated with various concentrations of CGA (0, 10, 50, and 100 μM) through the duration of maturation for 44 h. The treated oocytes were then fertilized, electro-stimulated at 30 V/mm with five 1 msec unipolar pulses, and subsequently cultured in vitro until development into the blastocyst stage. Without electro-stimulation, the treatment with 50 μM CGA had useful effects on the maturation rate of oocytes, the total cell number, and the apoptotic nucleus indices of blastocysts. When the oocytes were electro-stimulated after in vitro fertilization, the treatment with 50 μM CGA supplementation significantly improved the rate of oocytes that developed into blastocysts, but reduced the apoptotic nucleus indices (4.7% and 7.6%, respectively) compared with those of the untreated group (1.4% and 13.0%, respectively). These results suggested that

supplementation with 50 μM CGA during maturation improves porcine embryonic development and quality of electro-stimulation treated embryos.

Compared with other mammal counterpart, IVP system in pigs is less effective. However, our results indicated that the supplementation of 50 μM CGA during IVM has beneficial effects on embryonic development and quality of porcine embryos with or without electro-stimulation treatment. Moreover, the presence of CGA during oocyte maturation under heat stress effectively protects porcine oocytes from apoptosis and improved its developmental ability after parthenogenesis activation.

GENERAL INTRODUCTION

Pig as a research model

Traditional animal biomedical models such as zebra fishes as well as a various species of rodent have been widely used for decades as they are easy to be regulated in experimental facilities. These models also allow rapid and affordable basic genetic studies of specific genes effects on biological pathways related to human being. In some aspects, however, these models are not always good representative of the complexity of cells' physiology. There has been a growing awareness of the limitations of some certain animal models. Compared to the mouse model that have certainly contributed to our understanding of gene function and physiology, the pig model shows a greater similarity to humans in genome sequences as well as the anatomy and physiology. The pigs therefore offer many exciting applications including stem cell research, tissue engineering and xenotransplantation. The combination of new molecular biology methods, for examples, genome editing tools and reproductive technology such as in vitro embryo production and SCNT or cloning can now be applied to create genetically engineered pigs either in typical species or minipigs (Luo et al. 2012, Prather et al. 2013). To date, many genetically modified pig models with agricultural or biomedical values have been established by using gene editing tools. Genome editing tools including clustered, regularly interspaced, short palindromic repeats (CRISPR) together with CRISPR associated (Cas) nucleases (CRISPR/Cas), allow us to do precise manipulations in specific genes in several

organisms such as cells, zygotes and developing embryos (reviewed by Yang and Wu 2018).

The pig model is an appropriate medical model in human medicine for many reasons in terms of the similarity of weight and organ; physiology; genomic availability, transcriptomic, proteomic tools; the application of effective cloning and transgenic technologies and also the presence of stable cell lines (Critser et al. 2009). In recent years, advances in technologies applied to genetic engineering (Luo et al. 2012, Prather et al. 2013), transgenesis and the generation of knockouts in combination with somatic nuclear cloning, have provided the new opportunities to obtain new animal models mimicking major human diseases, to develop effective treatments. The pigs are considered one of the excellent animal models to provide a suitable source of cells and organs for xenotransplantation (Ramsoondar et al. 2009), and transgenic animals to produce the specific proteins relevant to human biology (Takahagi et al. 2005, Pan et al. 2010, Tanihara et al. 2016).

As the main source of food, proteins and fat in many geographical areas of the world, the pigs have been cultivated for centuries. It is now the time to take advantage of their similarities to humans to apply the powerful possibilities of transgenesis technologies in combination with the other omics on improving of human health.

IVP of pig embryos

IVP of mammalian embryos includes three major technical steps: i) IVM of oocytes recovered directly from follicles, ii) IVF or co-incubation of capacitated spermatozoa with in vitro matured oocytes, and iii) IVC of zygotes up to the certain embryonic stage such as the blastocyst stage. The porcine IVP embryo system is at great interest to produce large quantities of matured oocytes and embryos to be used not only in basic sciences such as physiology and reproduction, but also in advanced biotechnology and biomedical researches.

IVP in pigs have several advantages; it enables us to utilize a large number of immature oocytes that are normally lost through atresia process by using ovaries collected from slaughterhouses. During IVM the immature oocytes reach the MII stage that can be used as recipient oocytes for other reproductive technologies such as intracytoplasmic sperm injection (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 the possibility to produce a large number of embryos at lower costs within a shorter time. These advantages could help to increase the application of porcine IVP to further studies which depend on zygotes or blastocysts as source of materials. Recent development of gene editing technologies such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), or clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein

(Cas), are very effective in creating double-stranded breaks (DSB) at a specific locus of a genome, thereby facilitating genetic modifications, including knockouts via non-homologous end joining (NHEJ) and knock-ins via homology-directed repair (HDR) (Hsu et al. 2014). There technologies are mainly conducted by embryo injection of engineered nucleases into embryos and somatic cell nuclear transfer (reviewed by Yang and Wu 2018). Tanihara et al. (2016) have confirmed a new technique to perform gene editing by electroporation applied to zygotes which has the potential for facilitating genetic modification of pigs since this technology does not require the complicated methods associated with manipulators as in somatic cell reprogram or in ISCI does. To date, many genetically modified pig models with agricultural or biomedical values have been established by using gene editing tools. These pig models are expected to accelerate research progress in related fields and benefit humans in a future.

The first approach using IVP technologies in pigs was established when the porcine IVM oocytes to be fertilized (Motlik and Fulka, 1974). The first successful IVP embryos in pigs were reported four years later by Iritani et al. (1978). In 1988, Nagai et al. have successfully used the frozen-thawed boar spermatozoa to fertilize IVM pig oocytes. After that, the developmental competence of porcine oocytes to the blastocyst stage was first reported by Mattioli et al. (1989). Later, piglets were born after embryo transfer of IVP embryos at 2–4 cell stage (Yoshida et al. 1993), at the morula stage (Abeydeera

et al. 1998) or at the blastocyst stage were reported (Marchal et al. 2001, Kikuchi et al. 2002). Since then, a number of studies have shown that successful large-scale IVP of porcine embryos can provide viable embryos more efficiently, with less cost and in less time, when compared to the surgical collection of in vivo derived embryos from sows. For IVC, procedures have improved, however, for IVM and IVF systems still have several unsolved problems, including imbalance of nuclear and cytoplasmic maturation and polyspermy (Nagai et al. 2006, Dang Nguyen et al. 2011). These problems detain other reproductive techniques such as somatic cell nuclear transfer (SCNT), ICSI, genome editing, embryo transfer as well as establishment and utilization of embryonic stem cells that are essential for the production of transgenic animals and cells/organs for xenotransplantation. Thus, improving the competence of IVP embryos is crucial because the inefficiency of the IVP system would diminish its application on further studies, and because these techniques depend on blastocysts as source of materials.

Objectives of the study

As described above, IVP of embryos is a three-step methodology including IVM, IVF and IVC. According to reports (Lonergan and Fair, 2016), IVM is the key factor that determines the proportion of oocytes which develop to the blastocyst stage. Although substantial progress has been made to improve the efficiency of an IVM protocol, however, there is a lack of consistency in the success rate of conventional in vitro matured oocytes compared to the in vivo

counterpart. Multiple factors likely contribute to the overall poor quality of in vitro matured oocytes. Oxidative stress (OS) might be one of the important factors. 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). The generation of pro-oxidants such as ROS is an invariable phenomenon in the culture condition. In general, the high oxygen concentration associated with in vitro conditions, results in the generation of increased ROS and in turn, increased oxidative stress (Agarwal et al. 2003, Agarwal et al. 2006), which induces apoptosis (Kannan et al., 2000). On the other hand, ROS are considered signal molecules in oocyte physiology and their impact on maturation promoting factor (MPF) destabilization has recently been reported (Batista et al. 2016, Lin et al. 2015, Martinez et al. 2015).

Oocyte protection against ROS may play important roles in pre-implantation embryonic development. On the other hand, antioxidants are ROS scavengers, thereby helping to maintain the oocyte's oxidant/antioxidant balance. The effects of antioxidant supplementation to IVM media have been studied in various mammalian species (Ali et al. 2003, Li et al. 2007, Truong et al. 2017). A number of in vitro studies have demonstrated that both water-soluble (e.g., vitamin C) and lipid-soluble (e.g., vitamin E) substances categorized as antioxidants, as well as a variety of phenolic compounds (e.g., phenolic content, ascorbic acid and carotenoids), can help to prevent the oxidation process (Fernandez-Panchon et al. 2008). One of such phenolic compounds is

chlorogenic acid, (CGA, Fig. 1) which is formed by the esterification of caffeic acid and quinic acid. CGA is one of the most abundant polyphenolic component found in various agricultural products such as coffee, beans, potatoes and apples (Clifford 1999). Accumulating evidence has demonstrated that CGA exhibits many health benefits such as reduction of the relative risk of cardiovascular disease, diabetes type 2, and Alzheimer's disease (Lindsay et al. 2002, E et al. 2004, Ranheim and Halvorsen 2005) as well as anti-bacterial, anti-oxidant, and anti-carcinogenic activities (Kono et al. 1997, Kasai et al. 2000, Feng et al. 2005, Santos et al. 2006). However, information concerning about in vitro effects of CGA on oocyte and embryo development is remained unknown.

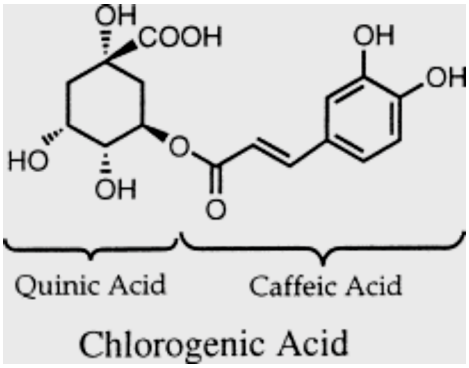


Fig. 1 Chemical structure of chlorogenic acid

The detrimental effects of oxidative stress on porcine oocytes and embryos 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 IVC media (Ozawa et al. 2006). In the present study, we focus on supporting

oocytes/embryos by reducing oxidative stress, which is a major detrimental effect of the IVC system on the developmental competence of porcine embryos by the present of CGA during porcine oocyte maturation. This study contains following three Chapters;

CHAPTER I: CGA supplementation during IVM improves maturation, fertilization and developmental competence of porcine oocytes.

CHAPTER II: Presence of CGA during IVM protects porcine oocytes from the negative effects of heat stress

CHAPTER III: Effects of CGA supplementation during IVM culture on the development and quality of porcine embryos with electro-stimulation treatment after IVF.

CHAPTER I:

CGA supplementation during IVM improves maturation, fertilization and development of porcine oocytes

INTRODUCTION

The IVP of porcine embryos is of interest to the scientific community because of its capacity to produce large quantities of matured oocytes and embryos that are crucial to basic science (such as reproductive physiology), as well as advances in biotechnology and biomedical research. Furthermore, with respect to anatomy and physiology, the pig is similar to humans and is therefore considered to be a suitable source of cells and organs for xenotransplantation (Critser et al. 2009, Taka et al. 2009, Samiec and Skrzyszowska 2011a) and expected as transgenic animal capable of producing specific proteins that it shares with humans (Pan et al. 2010, Samiec and Skrzyszowska 2011b, Takahagi et al. 2005). IVP systems comprise three major steps: IVM, IVF or SCNT and IVC of fertilized or cloned embryos (Samiec et al. 2015, Samiec and Skrzyszowska 2012a, Samiec and Skrzyszowska 2013, Somfai and Hirao 2011). Although many attempts have been made to produce high-quality IVF- or SCNT-derived embryos, their developmental competence in the pigs remains insufficient and lower than that of in vivo derived embryos of the pig and of other mammalian species, such as cattle or mice (Dang Nguyen et al. 2011, Kikuchi et al. 1999, Kikuchi et al. 2002, Samiec et al. 2012). Thus, it is crucial to improve the developmental competence of IVF- or SCNT-derived embryos because this inefficiency diminishes its application to further studies that require high-quality embryos (Pang et al. 2013, Samiec and Skrzyszowska 2012b, Samiec and Skrzyszowska 2014, Yoshioka et al. 2002). It has been established

that the oxygen concentration within the lumen of the female reproductive tract (in vivo) is approximately one-third that found under standard in vitro conditions (Mastroianni and Jones 1965). Generally, the high oxygen concentration associated with in vitro conditions results in increased generation of ROS and in turn increased oxidative stress on the oocytes (Agarwal et al. 2006, Agarwal et al. 2003). Therefore, antioxidants might be beneficial additives that could protect in vitro oocytes from stress and thereby improve developmental competence of oocytes. CGA is a quinic acid conjugate of caffeic acid (Gonthier et al. 2006) found at high levels in coffee beans and various sources of fruits including strawberries, blueberries, eggplants and tomatoes (Cho et al. 2010, Mahmood et al. 2012). As a phytochemical, several health benefits of CGA have been demonstrated, including antioxidative (Hoelzl et al. 2010), hepatoprotective (Xu et al. 2010), antiobesity (Cho et al. 2010), anti-inflammatory and antinociceptive effects (Kupeli et al. 2012). CGA appears to have similar antioxidant potential to caffeic acid, as assessed by the oxygen radical absorbance capacity (ORAC) (Ishimoto et al. 2012). In the present study, we investigated the antioxidant effects of CGA supplementation during IVM on the meiotic and developmental competence of porcine oocytes.

MATERIALS AND METHODS

As no live animals were used in this study, no ethical approval was required at any of the participating institutions.

IVM and assessment

Porcine ovaries were obtained from approximately 6-month-old gilts at a local slaughterhouse and were transported within 3 h to the laboratory in physiological saline at 30°C. 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 sulphate (Meiji). The COCs were collected from ovaries. The follicles of ovarian surface were sliced using a surgical blade on the sterilized dish. Only COCs with a uniformly dark-pigmented ooplasm and intact cumulus cell masses were collected under a stereomicroscope. 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; #12340, Invitrogen Co., Carlsbad, CA, USA), supplemented with 10% (v/v) porcine follicular fluid; 0.6 mM cysteine (Sigma-Aldrich, St. Louis, MO, USA); 50 μM sodium pyruvate (Sigma-Aldrich); 2 mg/ml D-sorbitol (Wako Pure Chemical Industries Ltd, Osaka, Japan); 1 μg/ml 17β-estradiol (Sigma-Aldrich); 10 IU/ ml equine chorionic gonadotropin (Kyoritsu Seiyaku, Tokyo, Japan); 10 IU/ml

human chorionic gonadotropin (Kyoritsu Seiyaku); and 50 μg/ ml gentamicin (Sigma-Aldrich), for 22 h in four-well dishes (Nunc A/S, Roskilde, Denmark). Subsequently, the COCs were transferred into maturation medium without hormone supplementation and cultured for an additional 22 h. The incubation of COCs was conducted at 39°C in a humidified incubator containing 5% CO2 in air. To assess the meiotic status of oocytes following IVM, some oocytes were denuded, fixed and permeabilized in Dulbecco's PBS (Invitrogen) supplemented with 3.7% (w/v) paraformaldehyde and 1% (v/v) Triton X-100 (Sigma-Aldrich) at 25°C for 15 min. Permeabilized oocytes were then placed on glass slides and stained with 1.9 mM bisbenzimide (Hoechst 33342; Sigma-Aldrich), before being covered with coverslips. After overnight incubation at 4°C, the oocytes were examined by fluorescence microscopy. Based on their chromatin configuration, they were classified as "germinal vesicle," "condensed chromatin," "metaphase I" or "metaphase II" (Wongsrikeao et al. 2004). Oocytes with the diffusely stained cytoplasmic characteristics of non-viable cells, and those in which chromatin was unidentifiable or not visible were classified as "degenerated.

IVF and assessment of fertilization status

IVF was performed according to methods described by 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., Yamagata, Japan) in a 15 ml test tube and were then washed by centrifugation at 500 x *g* for 5 min. The pellets of spermatozoa were resuspended in fertilization medium to obtain a final sperm concentration of 1×10^7 sperms/ml. Some of the spermatozoa (50 μl) were added to 50 μl of fertilization medium containing 10–20 matured oocytes. The final sperm concentration was adjusted to 5×10^6 sperms/ml. The oocytes were co-incubated with spermatozoa for 12 h at 39°C in a humidified incubator containing 5% CO2, 5% O2 and 90% N2. Following co-incubation with spermatozoa for 12 h, the presumed zygotes were denuded from the cumulus cells and attached spermatozoa by mechanical pipetting. To assess the fertilization of the oocytes, some 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 fertilized and were categorized as normal or polyspermic, based on the number of swollen sperm heads and/or pronuclei in the cytoplasm (Do et al. 2015).

IVC and assessment of blastocyst quality

The remaining denuded zygotes were subsequently transferred to 100 μl droplets of PZM-5 (Research Institute for the Functional Peptides Co.). Each

droplet contained approximately 10 presumed zygotes. The zygotes were cultured continuously in vitro at 39°C in a humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2. All of the cleaved embryos were transferred into 100 μl droplets of PBM (Research Institute for the Functional Peptides Co.) at 72 h after insemination and cultured for an additional 5 days to evaluate their ability to develop to the blastocyst stage. To evaluate the development stage of fertilized zygotes, all embryos were fixed on day 8 (day 0; insemination) and were stained with Hoechst 33342 to assess the quality of embryos by counting cell number.

Experimental design

Experiment 1

To evaluate the effects of CGA supplementation during IVM culture on the IVM, fertilization and development of porcine oocytes, the COCs were cultured in maturation medium supplemented with 10, 50, 100 and 200 μM CGA (Sigma-Aldrich). As a control, COCs were cultured in maturation medium without CGA. After maturation culture for 44 h, the COCs were fertilized in vitro and cultured continuously in vitro as described above.

Experiment 2

To assess the protective effect of CGA on hydrogen peroxide (H_2O_2)-induced DNA damage in porcine oocytes, the COCs were exposed to 1 mM H_2O_2

(Do et al. 2015) in maturation medium supplemented either with or without CGA (50 μM) during IVM. After maturation at 39°C for 44 h, the oocytes were denuded, fixed and then evaluated for nuclear status and DNA fragmentation, by a combined technique for simultaneous nuclear staining and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL), modified from procedures previously described by Otoi et al. (1999). Briefly, the oocytes were fixed overnight at 4°C in 3.7% (w/v) paraformaldehyde diluted in PBS. After fixation, the oocytes were permeabilized in PBS containing 0.1% (v/v) Triton X-100 for 40 min. They were subsequently incubated overnight at 4°C in PBS containing 10 mg/ml BSA (blocking solution). They were then incubated in fluorescein-conjugated 2-deoxyuridine 5-triphosphate and TUNEL reagent (Roche Diagnostics Corp., Tokyo, Japan) for 1 hr at 38.5°C. After TUNEL staining, the oocytes were counterstained with 1 μg/ml 4′6-diamidino-2-phenylindole (Invitrogen) for 10 min to assess the meiotic status of oocytes. They were then treated with an antibleaching solution (Slow-Fade; Molecular Probes Inc., Eugene, OR, USA), mounted on glass slides and then sealed with clear nail polish. Labelled oocytes were examined using an epifluorescence microscope (Eclipse 80i; Nikon). Apoptotic nuclei showed condensed and fragmented morphology (Brison and Schultz 1997, Pawlak et al. 2011). The apoptotic rate was calculated by dividing the number of oocytes containing DNA fragmented nuclei (labelled by TUNEL) by the total number of oocytes.

Experiment 3

To compare the supplementation effects of CGA and caffeic acid during IVM culture on the IVM, fertilization and development of porcine oocytes, the COCs were matured in the medium supplemented with 10, 50 and 100 μM caffeic acid (Sigma-Aldrich). As a control, COCs were cultured in maturation medium supplemented with 50 μM CGA. The concentration of CGA (50 μM) found most suitable for the development of embryos in Experiment 1 was used in this experiment.

Statistical analysis

Experiments were repeated five times for oocytes matured with CGA and four times for oocytes exposed to H_2O_2 and oocytes matured with caffeic acid. Percentages of matured oocytes, fertilized oocytes, monospermy, cleaved embryos, embryos develop to the blastocyst stage and apoptotic oocytes were subjected to arcsine transformation before ANOVA. The transformed data were tested by ANOVA, followed by Fisher's protected least significant difference test, using the StatView software (Abacus Concepts, Berkeley, CA, USA). Differences with a probability value (p) less than 0.05 or less were considered to be statistically significant.

RESULTS

Effects of CGA supplementation during IVM on oocyte maturation, fertilization and embryo development

As listed in Table 1.1, the maturation rate of oocytes matured with 50 μM CGA (78.8 ± 3.8%) was significantly increased, compared to the control (63.1 ± 3.0%) and two other CGA concentrations (64.6 ± 7.2% and 65.2 ± 4.7% for 10 and 200 μM, respectively) ($p < 0.05$). The fertilization rate (60.9 ± 4.3%) of oocytes matured with 50 μM CGA during IVM was significantly higher ($p < 0.05$) than that of oocytes matured without CGA (39.1 ± 1.9%). Moreover, the blastocyst formation rate of oocytes matured with 50 μM CGA (21.6 ± 2.2%)

was significantly higher ($p < 0.05$) than that of the control (9.3 ± 1.9%) and all other concentrations of CGA (13.9 ± 3.1%, 14.4 ± 2.9% and 12.2 ± 1.6% for 10, 100 and 200 μM, respectively). However, no effects of CGA treatment were observed on the monospermy and cleavage rates of embryos.

Effects of CGA supplementation during IVM on maturation rate and DNA fragmentation of porcine oocytes exposed to H_2O_2

As shown in Figure 1.1, exposure of oocytes to 1 mM H_2O_2 during IVM significantly reduced the maturation rate (1.4 ± 0.8%) compared to that of non-exposed oocytes (68.6 ± 2.9%) ($p < 0.01$). Supplementation of the maturation medium with 50 μM CGA significantly improved the maturation rate of oocytes exposed to H_2O_2 (46.2 ± 4.5%) ($p < 0.01$). When oocytes that had been matured without CGA were exposed to 1 mM H_2O_2 during IVM, the proportion of DNA-fragmented nuclei (62.9 ± 6.8%) was significantly higher ($p < 0.01$) than that of the nonexposed group (3.9 ± 1.8%). However, CGA treatment significantly reduced the proportion of DNA-fragmented nuclei (30.2 ± 5.9%) ($p < 0.05$).

Comparison of supplementation effects of caffeic acid during IVM on maturation, fertilization and development of porcine oocytes

As listed in Table 1.2, the rates of maturation, fertilization, monospermy and blastocyst formation of oocytes matured with 10 and 50 μM caffeic acid

were similar to the control oocytes matured with 50 μM CGA. In contrast, the rates of maturation, fertilization, monospermy and blastocyst formation of oocytes matured with 100 μM caffeic acid were significantly lower ($p < 0.05$) than those of the control oocytes. There were no differences in the cleavage rates of oocytes after IVF among the groups.

DISCUSSION

In the present study, we confirmed the antioxidant potential of CGA. Firstly, we found that supplementation of the maturation medium with 50 μM CGA significantly improved the rates of maturation, fertilization and blastocyst formation of oocytes. Furthermore, the effect of caffeic acid supplementation during IVM was similar to the CGA, which is a quinic acid conjugate of caffeic acid. Oxidative stress poses a threat to oocytes and embryos in vitro, when these cells are removed from their natural habitat into one that lacks maternal antioxidant factors. The oxygen concentration in a standard IVP system is guessed to be higher than that in the female reproductive tract (Mastroianni and Jones 1965). Moreover, high oxygen concentrations associated with in vitro conditions result in increased oxidative stress that has been reported to have negative effects on the quality of embryos and might lead to an early block and retardation of embryonic development (Agarwal et al. 2003, Agarwal et al. 2006). Considerable evidence in animal studies indicates that supplementation of

culture media with antioxidants, vitamins C and E, amino acids or ROS scavengers can be alternative treatment strategies that help to reduce oxidative stress and can be beneficial to embryonic survival and blastocyst formation rates (Taylor 2001). To date, a variety of media have been developed for the porcine IVP system. Each media system requires a balance between oxygen factors and antioxidants. Therefore, it is also necessary to investigate the optimal concentration of the supplemented antioxidant. Our results indicate that 50 μM is the optimal concentration of CGA supplementation during porcine IVM. To our knowledge, the present study was the first to employ this application of CGA to in vitro development of the porcine oocyte. CGA is a quinic acid ester of caffeic acid, which has the antioxidant ability with respect to their capability to quench a ROS (Foley et al. 1999). Rice-Evans et al. (1996) demonstrated that there are no differences between caffeic acid and CGA in their inhibitory effects on low-density lipoprotein oxidation. In this study, we demonstrated that the effects of 50 μM CGA supplementation to the maturation medium were comparable to 10 and 50 μM caffeic acid supplementation in porcine oocytes. It has demonstrated that 50 μM of caffeic acid protected human and mice cells against oxidative stress in vitro (Lapidot et al. 2002, Nardini et al. 1998). These studies support our results and the antioxidant ability of CGA.

The members of ROS family include H_2O_2 that is more stable than O_2 and can be readily diffused through cell membranes. A direct relationship between increased concentrations of H_2O_2 and apoptosis has been observed in fragmented

embryos and blastocysts (Lee and Yeung 2006, Yang et al. 1998). Pierce et al. (1991) have also identified H_2O_2 as a mediator of apoptosis in the blastocyst. Moreover, the maturation of oocytes and development of embryos are also affected by increased levels of ROS or reduced antioxidant defences (Blondin et al. 1997, Harvey et al. 2002). Therefore, apoptosis levels of oocytes and embryos can be useful indicators of oocyte quality and embryonic development (Brison and Schultz 1997, Tatemoto et al. 2000). In the present study, we confirmed the deleterious effects of H_2O_2 on the induction of DNA fragmentation in porcine oocytes matured in vitro that had been demonstrated in our previous study (Do et al. 2015). We also found that when oocytes were exposed to H_2O_2 during IVM, CGA effectively restored the oocyte maturation rate and protected oocytes from DNA fragmentation. These results confirmed the antioxidant effect of CGA on porcine oocytes exposed to H_2O_2 during IVM; therefore, CGA has a positive effect by preventing apoptosis and improving the quality of oocytes matured in vitro. Other antioxidants and H_2O_2 scavengers also have a positive effect on preventing H_2O_2 damage during porcine IVP. Melatonin is one of the antioxidants and free radical scavengers, which has beneficial effects on nuclear and cytoplasmic maturation during porcine IVM (Kang et al. 2009). Yazaki et al. (2013) have reported l-carnitine, an H_2O_2 scavenger, improves H_2O_2-induced impairment of nuclear maturation in porcine oocytes. Vitamin-E is also one of the antioxidants. Vitamin-E suppressed oxidative damage and improved their developmental ability of porcine oocytes (Kitagawa et al. 2004). These studies

support the antioxidant effect of CGA on the improvement in porcine IVP system.

In conclusion, CGA is an effective antioxidant that improves the maturation, fertilization and developmental competence of porcine IVP oocytes and protects oocytes from DNA fragmentation caused by H_2O_2 exposure. CGA also has comparable effects to caffeic acid on improving IVP of porcine embryos. It appears that CGA supplementation in the maturation medium improves the porcine IVP system, which can be beneficial to further developments in biotechnology.

Table 1.1. The effects of chlorogenic acid (CGA) supplementation during in vitro maturation culture on the maturation, fertilization and development of porcine oocytes[a].

Concentration of CGA (μM)	Number of examined oocytes	Number (%)[b] of matured oocytes	Number of examined oocytes	Number of oocytes		Number of examined oocytes	Number of oocytes	
				Fertilized (%)	Monospermy (%)[c]		Cleaved (%)	Developed to blastocysts (%)
0	144	93 (63.1 ± 3.0)[a]	92	37 (39.1 ± 1.9)[a]	25 (74.5 ± 7.8)	209	157 (75.7 ± 4.7)	19 (9.3 ± 1.9)[a]
10	133	93 (64.6 ± 7.2)[a]	112	58 (54.0 ± 4.0)[ab]	36 (60.7 ± 5.5)	223	179 (80.6 ± 4.3)	30 (13.9 ± 3.1)[a]
50	151	118 (78.8 ± 3.8)[b]	108	63 (60.9 ± 4.3)[b]	42 (78.9 ± 2.9)	211	182 (86.7 ± 1.3)	45 (21.6 ± 2.2)[b]
100	147	112 (75.9 ± 2.5)[a,b]	95	45 (49.8 ± 7.9)[ab]	24 (64.8 ± 6.2)	216	182 (84.5 ± 3.4)	30 (14.4 ± 2.9)[a]
200	151	104 (65.2 ± 4.7)[a]	97	44 (47.2 ± 4.8)[ab]	23 (64.0 ± 6.0)	223	175 (78.7 ± 1.4)	27 (12.2 ± 1.6)[a]

[a]Five replicated trials were carried out.

[b]Percentages are expressed as mean ± SEM.

[c]The monospermic fertilization rate was defined as a ratio of the number of monospermic oocytes to the total number of fertilized oocytes.

[a,b]Values with different superscripts in the same column differ significantly ($p < 0.05$).

Table 1.2. The effects of caffeic acid supplementation during in vitro maturation culture on the maturation, fertilization and development of porcine oocytes[a].

Concentration of caffeic acid (μM)	Number of examined oocytes	Number (%)[b] of matured oocytes	Number of examined oocytes	Number of oocytes		Number of examined oocytes	Number of oocytes	
				Fertilized (%)	Monospermy (%)[c]		Cleaved (%)	Developed to blastocysts (%)
0[d]	54	40 (73.9 ± 2.7)[a]	60	44 (73.3 ± 4.7)[a,b]	37 (61.7 ± 1.7)[a,b]	145	130 (89.7 ± 1.5)	14 (9.7 ± 1.8)[a]
10	52	37 (71.2 ± 1.9)[a,b]	59	42 (71.2 ± 1.6)[a,b]	37 (62.7 ± 1.7)[a]	150	133 (88.7 ± 2.1)	11 (7.3 ± 2.1)[a,b]
50	52	40 (76.9 ± 3.1)[a]	58	11 (75.9 ± 4.4)[a]	38 (65.5 ± 2.8)[a]	139	124 (89.2 ± 1.1)	6 (4.3 ± 2.6)[a,b]
100	55	35 (63.98 ± 2.0)[b]	57	35 (61.4 ± 3.4)[b]	31 (54.7 ± 3.2)[b]	143	121 (84.6 ± 2.0)	4 (2.8 ± 1.5)[b]

[a]Five replicated trials were carried out.

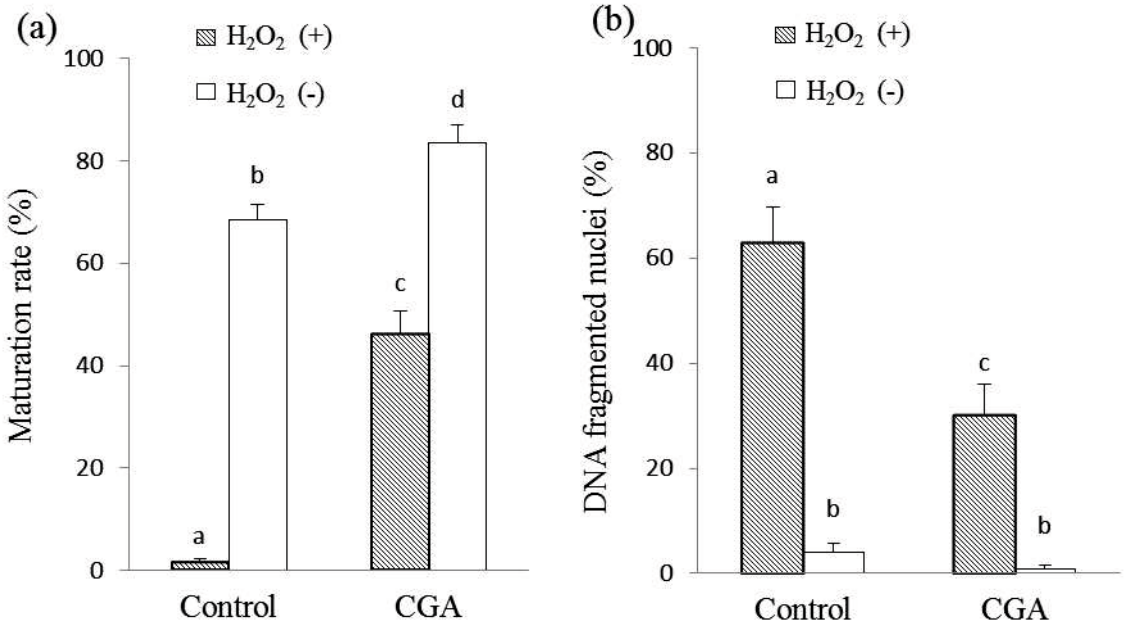
[b]Percentages are expressed as mean ± SEM.

[c]The monospermic fertilization rate was defined as a ratio of the number of monospermic oocytes to the total number of fertilized oocytes.

[c]The monospermic fertilization rate was defined as a ratio of the number of monospermic oocytes to the total number of fertilized oocytes.

[a,b]Values with different superscripts in the same column differ significantly ($p < 0.05$).

Fig. 1. 1. Eeffects of chlorogenic acid (CGA; 50 μM) supplementation during in vitro maturation on the maturation rate (a) and the proportion of DNA-fragmented nuclei (b) of porcine oocytes exposed to 1.0 mM H_2O_2. Oocytes matured without CGA served as control group. Each bar presents the mean value ± SEM (n = 4 replications, each with 100 – 110 oocytes per treatment). Bars with different letters differ significantly ($p \leq 0.05$).



CHAPTER II

Presence of CGA during IVM protects porcine oocytes from the negative effects of heat stress

INTRODUCTION

Heat stress is an important issue in the pork industry as it greatly impairs the reproductive performance of sows and thus has economic consequences (Tast et al. 2002, Bertoldo et al. 2010). Heat stress compromises female fertility because the development of gametes and embryos is diminished under high ambient temperature (Ross et al. 2015), leading to impairment of follicle development, oocyte maturation, embryo development and fetal growth (Fu et al. 2014). It has been reported that heat stress has detrimental effects on oocyte cytoskeletal organization and meiotic and development competence when porcine ovaries or oocytes are exposed to elevated temperatures (Ju et al. 2004, Barati et al. 2008, Tong et al. 2004). Heat stress during oocyte maturation activates the pathway of apoptosis, which is mediated by group II caspases (Roth and Hansen 2004). Moreover, heat stress increases oxidative stress within cells through accumulation of ROS, which are key players in embryonic developmental failure (Sakatani et al. 2008, Matsuzuka et al. 2005, Agarwal et al. 2012). Excessive levels of ROS are extremely disruptive to cellular function through interaction with lipids, proteins and nucleic acids, causing loss of membrane integrity, structural and functional changes to proteins, and damage to nucleic acids (Tamura et al. 2012).

Antioxidant supplementation can prevent or reduce intracellular ROS accumulation and improve the developmental competence of embryos (Camano et al. 1998, Maya-Soriano et al. 2013). CGA is a phenolic compound found mainly in coffee and tea, as well as in many fruits and vegetables (Gonthier et al.

2006, Mahmood et al. 2012). Previously, it has been demonstrated that CGA has ROS-scavenging, antioxidant, and antiapoptotic activity (Wu et al. 2012), as well as acting as a defence factor to protect porcine oocytes from oxidative stress induced by hydrogen peroxide and electro-stimulation conditions (Nguyen et al. 2017, Nguyen et al. 2018). Furthermore, CGA has been reported to have low cytotoxic effects on cellular proliferation when the cells were treated with different concentrations of CGA (Jin et al. 2005).

On this basis, we have hypothesized that CGA can alleviate the effects of heat stress on porcine oocytes. In this study, we investigated the effects of CGA supplementation during porcine oocyte IVM under both hyperthermic (41.0°C) and isothermic (38.5°C) conditions, focusing on nuclear maturation, apoptosis status and embryonic developmental competence after parthenogenetic activation.

MATERIALS AND METHODS

As no live animals were used in this study, no ethical approval was required at any of the participating institutions.

Oocyte collection and IVM

Porcine ovaries were obtained from prepubertal crossbred gilts (Landrace × Yorkshire breeds) at a local slaughterhouse and transported to the laboratory within 1 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; Sigma-

Aldrich Co., St. Louis, MO, USA) supplemented with 100 IU/mL penicillin G potassium (Meiji Seika Pharma Co., Ltd. Tokyo, Japan) and 0.1 mg/mL streptomycin sulfate (Meiji, Tokyo, Japan). COCs were collected by dissecting follicles 2-6 mm in diameter in Medium 199 with Hank's salts (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 20 mM HEPES. After dissection, only COCs with uniformly dark-pigmented ooplasm and intact cumulus cell masses were collected. The basic medium for IVM was previously defined porcine oocyte medium (POM) (Yoshioka et al. 2008) supplemented with 3 mg/mL polyvinyl alcohol (PVA) and 10 ng/mL epidermal growth factor (eGF; Sigma-Aldrich). During the first 22 h of IVM, 10 IU/mL equine chorionic gonadotropin (eCG; Serotropin, ASKA Pharmaceutical Co., Ltd., Tokyo, Japan), 10 IU/mL human chorionic gonadotropin (hCG, 500 units; Puberogen, Novartis Animal Health, Tokyo, Japan) and 1.0 mM/L dibutyryl cyclic adenosine monophosphate sodium salt (dbcAMP; Sigma-Aldrich) were added to the IVM medium (POM 1). At 22 h of IVM, the groups of oocytes were washed three times in the same medium without hormones and dbcAMP (POM 2) and cultured in POM 2 for an additional 22-24 h of IVM. Approximately 50 COCs were cultured in 500 μl of maturation medium under mineral oil (Sigma-Aldrich) in 4-well culture dishes (SPL, Gyeonggi, Korea). All incubations were performed in a humidified incubator containing 5% CO_2, 5% O_2 and 90% N_2 in air at 38.5°C.

Analysis of oocyte nuclear maturation and apoptosis status

To evaluate the rates of oocyte maturation and apoptosis, the oocytes were analyzed after IVM using a combined technique for simultaneous nuclear and TUNEL staining modified from procedures described previously (Do et al. 2015). Briefly, the oocytes were freed from cumulus cells by exposure to 150 IU of hyaluronidase (Sigma-Aldrich) and mechanical pipetting. Denuded oocytes 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, then incubated overnight at 4°C in PBS containing 10 mg/mL BSA (blocking solution). They were then incubated in fluorescein-conjugated 2-deoxyuridine 5-triphosphate and TUNEL reagent (Roche Diagnostics Corp., Basel, Switzerland) for 1 h at 38.5°C. The oocytes were then placed on glass slides and counterstained with 1.9 mM bisbenzimide (Hoechst 33342; Sigma-Aldrich) before being overlaid with coverslips and sealed with clear nail polish. The labeled oocytes were examined using an epifluorescence microscope (BX53; Olympus, Tokyo, Japan) under excitation wavelengths of 550 nm and 350 nm to visualize the TUNEL and Hoechst staining, respectively. Any oocyte with a green TUNEL-labeled nucleus was classified as apoptotic (Figure 1).

Parthenogenetic stimulation of IVM oocytes

Parthenogenetic stimulation of IVM oocytes was conducted as described by Iwamoto et al. (2005). Briefly, denuded oocytes without cumulus cells were transferred to activation solution consisting of 0.28 M D-mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$ and 0.01% (w/v) BSA and washed once. The oocytes were then stimulated with a direct current pulse of 1.5 kV/cm for a duration of 100 μsec using a somatic hybridizer (LF500G1; BEX, Tokyo, Japan) connected to a LF101 cell fusion unit (BEX, Tokyo, Japan).

Embryo culture and assessment of blastocyst quality

After electro-stimulation, the oocytes were washed with pig zygote medium 3 (PZM3; Yoshioka et al. 2002) and cultured in 50-μl droplets of PZM3 overlaid with mineral oil in 35-mm petri dishes for 7 days. On Day 2 (Day 0 = the day of stimulation), the culture dishes were supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma-Aldrich). Cleavage and blastocyst formation were evaluated under a stereo microscope on Day 2 and Day 7, respectively.

To evaluate the total number of cells per blastocyst, embryos on Day 7 were fixed and permeabilized in Dulbecco's PBS (DPBS; Invitrogen) supplemented with 3.7% (w/v) paraformaldehyde and 1% (v/v) Triton X-100 (Sigma-Aldrich) at room temperature for 15 min. The embryos were then placed on glass slides, stained with 1.9 mM Hoechst 33342 diluted in glycerol, and mounted under coverslips. The slides were kept at 4–8 °C overnight and then the embryos were examined by fluorescence microscopy (Figure 2). In the present

study, any embryo having a clear blastocoel and consisting of more than 16 cells was defined as a blastocyst.

Experimental design

Experiment 1

To evaluate the effects of CGA supplementation on nuclear maturation and apoptosis status of oocytes during IVM under hyperthermic or isothermic conditions, COCs were matured at either 41.0°C or 38.5°C, respectively, in maturation medium supplemented with 0 (as a control) or 50 μM CGA (Sigma-Aldrich). The concentration of CGA employed was that shown to significantly improve maturation and embryo development in the previous experiment (Nguyen et al. 2017, Nguyen et al. 2018). After IVM, the nuclear maturation and apoptosis status of oocytes were analyzed as described above.

Experiment 2

To evaluate the effects of CGA supplementation during IVM under hyperthermic or isothermic conditions on subsequent blastocyst formation ability, oocytes were matured at either 41.0°C or 38.5°C, respectively, in maturation medium supplemented with 0 (control group) or 50 μM CGA. After IVM, the oocytes were parthenogenetically activated and then cultured as described above. Embryo development and blastocyst quality were assayed as described above.

Experiment 3

To clarify the antioxidant effects of CGA, oocytes were matured under isothermic conditions at 38.5°C in POM medium supplemented with 0 (control group) or 50 μM CGA but without hypotaurine, L-cysteine and eGF, which may act as potential antioxidants. The rates of oocyte maturation and apoptosis were then compared between the two groups, as described for Experiment 1.

Statistical analysis

Each experiment was replicated at least four times. Data for oocyte maturation, apoptotic nuclei, and cleavage and development of embryos to the blastocyst stage expressed as percentages, as well as the total numbers of cells per blastocyst, were subjected to arcsine transformation before analysis. The transformed data were tested by ANOVA, followed by protected Fisher's least significant difference test, using StatView (Abacus Concepts, Berkeley, CA, USA). Differences at $p < 0.05$ were considered statistically significant.

RESULTS

Effects of CGA supplementation on oocyte maturation under hyperthermic conditions

The effects of CGA supplementation during IVM on oocyte maturation and apoptosis are shown in Table 2.1. When porcine oocytes were exposed to

41.0°C during IVM, the maturation rate (69.3%) was significantly lower than that of oocytes matured at 38.5°C (80.4%). Supplementation of the maturation medium with 50 μM CGA significantly improved the maturation rate of the oocytes at 41.0°C (79.6%) relative to that of oocytes matured in the absence of CGA (69.3%). Exposure to hyperthermic conditions during IVM induced apoptosis in porcine oocyte nuclei regardless of the presence of CGA in the maturation medium. The rates of apoptosis of oocytes matured at 41.0°C in maturation medium supplemented with and without CGA were 8.6% and 18.6%, respectively, and they were significant different. However, no apoptosis was observed when oocytes were matured at 38.5°C. Supplementation of the medium with 50 μM CGA resulted in a significantly lower apoptosis rate relative to that of oocytes matured without CGA.

Effects of CGA supplementation during IVM under hyperthermic conditions on subsequent embryo development and blastocyst quality after parthenogenetic activation

When porcine oocytes were matured at 38.5°C, supplementation of the IVM medium with 50 μM CGA did not affect the proportion of cleaved embryos; 70.6% for oocytes matured with CGA and 69.0% for oocytes matured without CGA, but significantly increased the blastocyst formation rate (17.2%) compared with that of oocytes matured without CGA (7.7%). However, there was no significant difference in the number of cells per blastocyst between the control (0

μM) and 50 μM CGA groups matured at 38.5°C. When oocytes were matured at 41.0°C, supplementation of IVM medium with 50 μM CGA did not affect the proportion of cleaved embryos; 64.0% for oocytes matured with CGA and 61.1% for oocytes matured without CGA, but tended to increase ($p < 0.1$) the rate of blastocyst formation (12.0%) compared with that of the control group (5.3%)(Table 2.2). Furthermore, the mean number of cells in embryos generated from oocytes matured in the presence of CGA (47.7 cells) was higher than that in the control group (32.1 cells).

Antioxidant effects of CGA on oocyte maturation and apoptosis

To demonstrate unequivocally that CGA exerts antioxidant effects during porcine oocyte maturation, we performed IVM using POM medium without L-cysteine, hypotaurine and eGF at 38.5°C. Under these conditions, the oocyte maturation rate dropped to 59.0%; however, supplementation of the medium with 50 μM CGA significantly improved the maturation rate to 77.9% (Table 2.3).

DISCUSSION

Several studies have demonstrated that heat stress (culture under hyperthermic conditions) exerts detrimental effects on oocytes and their subsequent embryo development, and that supplementation of growth media with various antioxidants has the potential to improve their developmental competence (Roth 2015, Nabenishi et al. 2012, Balboula et al. 2013, Tseng et al.

2006, Isom et al. 2007, Ju et al. 2004, Do et al. 2015). Moreover, it has been reported that heat stress affects not only cell function directly (Roth 2015), but also induces accumulation of oxidizing agents such as ROS, causing DNA damage, lipid peroxidation, and disruption of mitochondrial function, leading in turn to abnormal gene expression and protein synthesis, and finally resulting in cell death (Loven 1988, Lord-Fontaine and Averill-Bates 2002).

In the present study we investigated the effect of CGA, a natural antioxidant, on the maturation and apoptosis status of porcine oocytes under heat stress and assayed their subsequent embryonic development. When porcine oocytes were matured under isothermic conditions, supplementation of the IVM medium with 50 μM CGA had no effect on oocyte maturation or the percentage of apoptotic oocytes. At this temperature, the oocyte maturation rate was approximately 80%, and no apoptotic oocytes were detected. On the other hand, when oocytes were matured under hyperthermic conditions, the maturation rate dropped significantly, accompanied by a dramatic increase of apoptosis. Under hyperthermic conditions, supplementation of the IVM medium with 50 μM CGA restored the oocyte maturation rate to roughly that obtained under isothermic conditions. Furthermore, under hyperthermic conditions, CGA significantly reduced the percentage of apoptotic oocytes. These results confirm that heat stress has a negative impact on the nuclear maturation of porcine oocytes, triggering apoptosis in them, and that both of these effects can be alleviated in the presence of 50 μM CGA.

Experiment 2 demonstrated that when porcine oocytes were matured at 38.5°C, supplementation of the IVM medium with 50 μM CGA significantly improved the potential of oocytes to develop to blastocyst-stage embryos after parthenogenetic activation, thus confirming our previous results for IVF-derived and electro-stimulated porcine embryos (Nguyen et al. 2017, Nguyen et al. 2018). Nevertheless, when applied at 38.5°C, CGA had no effect on the quality of the resulting blastocysts, as assessed in terms of total cell number. When oocytes were matured under hyperthermic conditions, CGA treatment only tended ($p<0.1$) to improve blastocyst development. On the other hand, hyperthermia during oocyte maturation caused a significant reduction in the number of cells in the blastocysts that subsequently developed, and this was successfully prevented by addition of CGA to the maturation medium. These results demonstrate that during oocyte maturation heat stress negatively impacts the quality of subsequently developing embryos, and that this can be alleviated by adding 50 μM CGA to the medium during IVM.

Chemically, CGA is a natural compound formed between caffeic acid and quinic acid. CGA and caffeic acid have vicinal hydroxyl groups on an aromatic residue, and exhibit antimutagenic, anticarcinogenic and antioxidant activities in vitro, which play a major role in ROS scavenging (Rice-Evans et al. 1996). Heat stress is known to exert detrimental effects on oocytes through increased intracellular accumulation of ROS, leading to oxidative stress (Sakatani et al. 2008; Matsuzuka et al. 2005; Agarwal et al. 2012). Previously we have

demonstrated that CGA exerts antioxidant effects on porcine oocytes during exposure to hydrogen peroxide (Nguyen et al. 2017). In experiment 3, depletion of all potential antioxidants from the IVM medium significantly reduced the oocyte maturation rate even at 38.5°C, which in turn was prevented by addition of 50 μM CGA to the medium. This suggests that restoration of the oocyte maturation rate was attributable to the antioxidant action of CGA. Since ROS accumulation is a frequent cause of apoptosis in cultured animal cells (Guerin et al. 2001), this effect can explain the suppression of apoptosis by CGA in Experiment 1.

Taken together, the present results suggest that CGA is an effective antioxidant for alleviating the negative effects of heat stress on porcine oocytes and subsequent embryo development. In comparison with other mammalian species, porcine oocytes and embryos are more sensitive to elevated temperature because of their high cellular lipid content (Ross et al. 2015). In both the northern and southern hemispheres, heat stress during the hot seasons has been reported to reduce the efficacy of in vitro embryo production in pigs by affecting the quality of follicular oocytes (Suzuki et al. 2010; Bertoldo et al. 2010). Further research will be needed to clarify whether the potential use of CGA might overcome this problem.

In conclusion, we have shown that CGA effectively protects porcine oocytes from the negative effects of heat stress during IVM by reducing the rate of apoptosis, thus improving both oocyte maturation and embryo quality.

Table 2.1. Effects of chlorogenic acid (CGA) supplementation during in vitro maturation on porcine oocyte status under isothermic and hyperthermic conditions.[*]

Exposure temperature (°C)	Treatment	No. of examined oocytes	Number (%) of matured oocytes	Number (%) of apoptotic oocytes[**]
38.5 (isothermic)	POM + CGA 50 μM	226	189 (83.6 ± 1.6)[a]	0[a]
	POM w/o CGA	209	168 (80.4 ± 2.9)[a]	0[a]
41.0 (hyperthermic)	POM + CGA 50 μM	162	129 (79.6 ± 1.9)[a]	14 (8.6 ± 3.0)[b]
	POM w/o CGA	231	160 (69.3 ± 2.1)[b]	43 (18.6 ± 4.4)[c]

[*]Six replicate trials were carried out. Data are expressed as the mean ± SEM.

[**]The apoptosis index was defined as the ratio of the number of oocytes containing an apoptotic nucleus to the total number of oocytes examined.

[a,b,c]Values with different superscripts in the same column are significantly different ($p < 0.05$).

Table 2.2. Effects of CGA supplementation during in vitro maturation of porcine oocytes under isothermic and hyperthermic conditions on their subsequent development after parthenogenetic activation.

Exposure temperature (°C)	Treatment	No. of examined embryos	Number (%) of cleaved embryos	Number (%) developing to blastocyst stage	Total number of cells per blastocyst
38.5 (isothermic)	POM + CGA 50 μM	262	185 (70.6 ± 5.2)	45 (17.2 ± 2.9)[a]	52 ± 2.2[a]
	POM w/o CGA	297	205 (69.0 ± 4.0)	25 (7.7 ± 2.5)[b]	46.0 ± 3.2[a]
41.0 (hyperthermic)	POM + CGA 50 μM	283	181 (64.0 ± 2.0)	34 (12.0 ± 2.1)[ab]	47.7 ± 2.3[a]
	POM w/o CGA	303	185 (61.1 ± 3.4)	16 (5.3 ± 1.5)[b]	32.1 ± 3.4[b]

[*]Six replicate trials were carried out. Data are expressed as the mean ± SEM.

[a,b]Values with different superscripts in the same column are significantly different ($p < 0.05$).

Table 2.3. Effects of CGA on maturation and apoptosis rates of porcine oocytes cultured in medium lacking hypotaurine, L-cysteine and eGF[*].

Treatment	No. of examined oocytes	Number (%) of matured oocytes	Number (%) of apoptotic oocytes
POM[**] + CGA 50μM	199	155 (77.9 ± 3.8)[a]	0
POM[**]	195	115 (59.0 ± 2.5)[b]	2 (0.9 ± 0.5)

Oocytes were cultured at 38.5°C (under isothermic conditions).

[*]Four replicate trials were carried out. Data are expressed as the mean ± SEM.

[**]Hypotaurine, L-cysteine and eGF were excluded from the original POM medium.

[a,b]Values with different superscripts in the same column are significantly different ($p < 0.05$).

Figure 2.1. Representative images of porcine oocytes exposure to bright field, Hoechst 33342 and TUNEL. Blue and green colours represent regular nuclei and apoptotic nuclei, respectively. Scale bars = 50 μm.

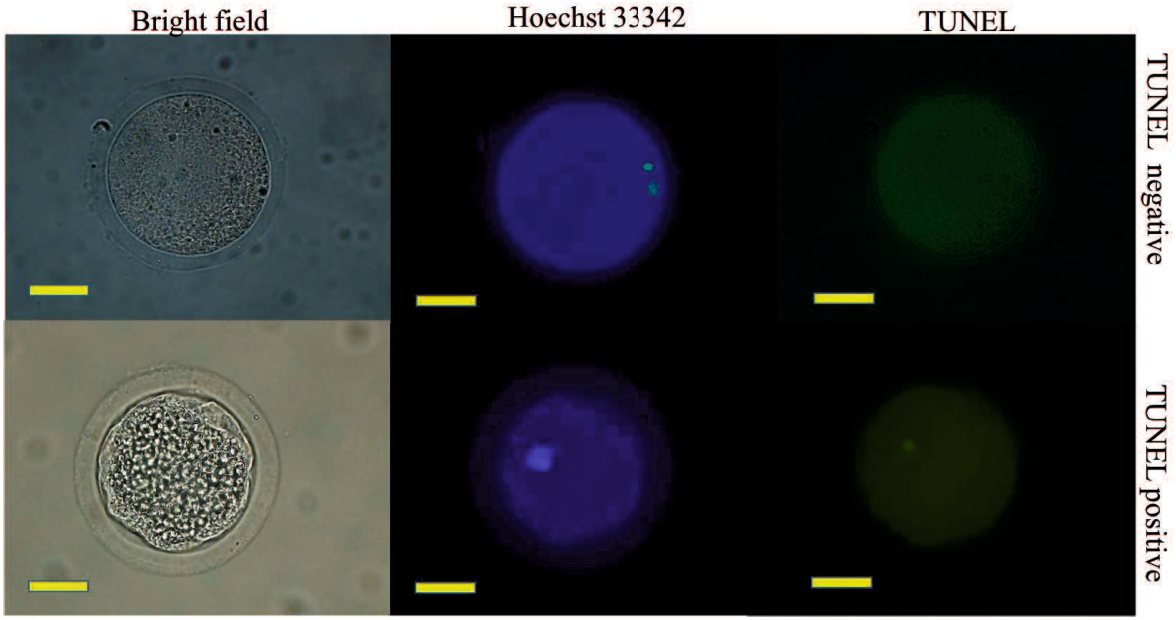
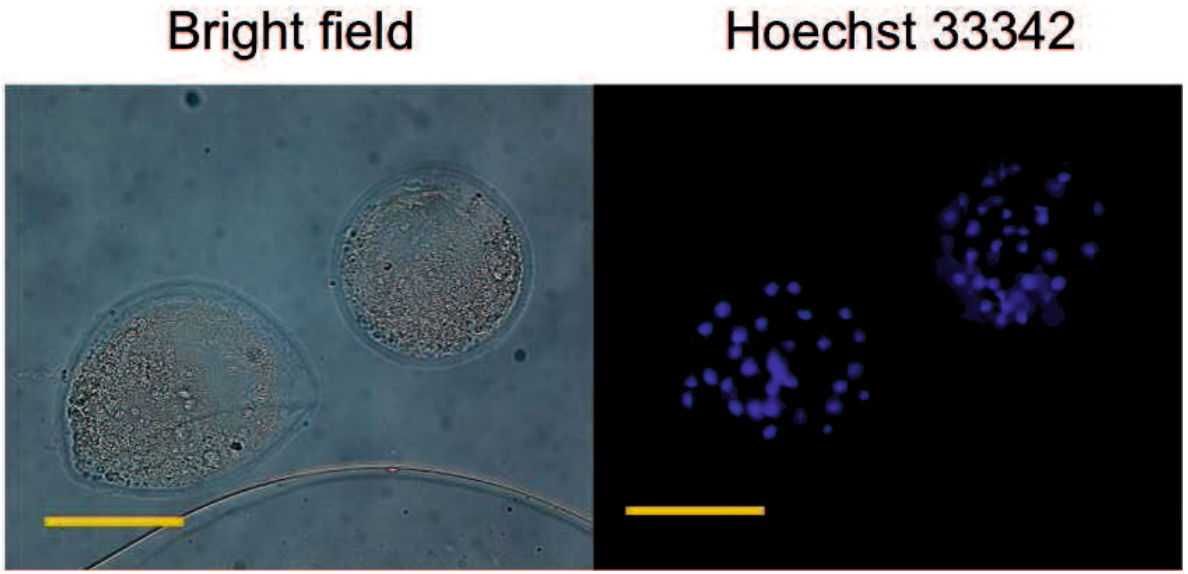


Figure 2.2. Representative images of porcine blastocysts exposure to bright field and Hoechst. The number of cell in each blastocyst was calculated by the number of blue-labeled nuclei with Hoechst 33342. Scale bars = 100 μm.



CHAPTER III

Effects of chlorogenic acid supplementation during IVM on the development and quality of porcine embryos with electro-stimulation treatment after IVF

INTRODUCTION

A transgenic animal model is a powerful tool for developing a more detailed understanding of gene regulation and function in biological systems (Boverhof et al. 2011). This technology introduces an exogenous gene into the embryonic cells' genomes, where it is expressed and inherited by offspring. Some techniques, such as microinjection, somatic cell nuclear transfer (SCNT), and sperm-mediated gene transfer (SMGT), are available for the production of transgenic animals, and each is of value in certain circumstances. Over the last three decades, microinjection has been widely accepted as the gold standard to transfer the exogenous gene into the animal genome at random sites (Garrels et al. 2011, Li et al. 2014, Meyer et al. 2010, Proudfoot et al. 2015, Wongsrikeao et al. 2011, Yang et al. 2013). Pigs (Sus scrofa) have been recognized as an important model organism for several biomedical types of research, including animal transgenesis, since they exhibit anatomical and physiological commonalities almost identical to humans (Ramsoondar et al. 2009, Samiec and Skrzyszowska 2011). Although the microinjection of exogenous genes into the porcine zygotes resulted in successful production of transgenic piglets with an acceptable germline transmission rate to their offspring (Garrels et al. 2011, Ivics et al. 2014, Li et al. 2014), the involvement of skilled personnel and extended periods of micromanipulation causing severe embryonic damage are a major limitation of this technique (Iqbal et al. 2009). Electroporation has thus become an alternative technique due to its ease of use and sufficient embryonic survival rate (Kaneko et al. 2014)

The technique of electroporation has been primarily utilized to introduce foreign DNA into a donor cell for use in producing transgenic animals by SCNT (Ross et al. 2010). It later was used successfully to create gene knockout and gene knock-in mice and rats by direct delivery of a recent high-impact materials called “engineered endonucleases” into mouse or rat embryos for the production of transgenic animals (Kaneko and Mashimo 2015, Kaneko et al. 2014). However, the mechanism underlying membrane electropermeabilization is still unknown. It has been suggested that electroporation under conditions compatible with cell survival induces lipid hydroperoxide formation in the cell membranes (Maccarrone et al. 1995). The production of hydroperoxides leads to the formation of pores by local membrane disaggregation in lipid bilayers. A further product of electroporation-induced lipid peroxidation is singlet oxygen (Maccarrone et al. 1995). Since free radicals are formed by the oxidative modification of the cell membrane, it is possible that oxidative damage may occur in some cellular structures. Therefore, the use of electroporation may induce cellular stress that results in the accumulation of ROS in embryonic cell cytoplasm (Maccarrone et al. 1995; Shil et al. 2005). Overproduction of ROS under various cellular stresses can lead to embryonic death (Agarwal et al. 2006; Agarwal et al. 2003). Antioxidant defence systems can regulate ROS generation and relieve the toxic effects while improving the developmental competence of embryos. Chlorogenic acid (CGA) is an ester of caffeic acid and quinic acid that is found mostly in coffee beans in addition to many plant compounds (Gonthier

et al. 2006, Mahmood et al. 2012). It exhibits several health benefits, including antioxidant (Hoelzl et al. 2010), hepatoprotective (Xu et al. 2010), anti-obesity (Cho et al. 2010), anti-inflammatory and antinociceptive effects (Kupeli et al. 2012). Moreover, it has been demonstrated that CGA limits apoptosis related to oxidative stress by a reduced ROS production and by an increase of intracellular glutathione levels in a human hepatoma cell line (Granado-Serrano et al. 2007). In this study, we therefore evaluated the protective effects of various concentrations of CGA on the developmental competence of electro-stimulation treated porcine embryos derived from oocytes matured in vitro. This study can be very beneficial in establishing the feasibility of porcine transgenesis technology for the future.

MATERIALS AND METHODS

There were no live animals used in this study, so no ethics approval was required.

IVM and assessment

Porcine ovaries were obtained from approximately 6-month-old gilts at a local slaughterhouse and were transported within 1 hr to the laboratory in physiological saline at 30°C. Ovaries were placed in 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 sulphate (Meiji). The follicles on the ovarian surface were sliced using a surgical blade on a sterilized dish. Only cumulus-oocyte complexes (COCs) with a uniformly dark-pigmented ooplasm and intact cumulus cell masses were collected under a stereomicroscope. Approximately 50 COCs were then cultured in 500 μl of maturation medium, consisting of 25 mM/L HEPES tissue culture medium 199 with Earle's salts (TCM 199; Invitrogen Co., Carlsbad, CA, USA) supplemented with 10% (v/v) porcine follicular fluid, 50 μM/L sodium pyruvate (SigmaAldrich, St. Louis, MO, USA), 2 mg/ml D-sorbitol (Wako Pure Chemical Industries Ltd., Osaka, Japan), 10 IU/ml equine chorionic gonadotropin (Kyoritu Seiyaku, Tokyo, Japan), 10 IU/ml human chorionic gonadotropin (Kyoritu Seiyaku), and 50 μg/ml gentamicin (SigmaAldrich) for 22 h in four-well dishes (Nunc A/S, Roskilde, Denmark). Subsequently, the COCs were transferred into maturation medium without hormone supplementation and cultured for an additional 22 hr. The incubation of COCs was conducted at 39°C in a humidified incubator containing 5% CO2 in air.

To assess the meiotic status of oocytes following IVM, some oocytes were denuded, fixed, and permeabilized in Dulbecco's PBS (DPBS; Invitrogen), supplemented with 3.7% (w/v) paraformaldehyde and 1% (v/v) Triton X-100 (Sigma-Aldrich) at 25°C for 15 min. Permeabilized oocytes were then placed on

glass slides and stained with 1.9 mM/L bisbenzimide (Hoechst 33342; Sigma-Aldrich) before being covered with coverslips. After overnight incubation at 4°C, the oocytes were examined by fluorescence microscopy. Based on their chromatin configuration, they were classified as “germinal vesicle”, “metaphase I”, or “metaphase II” (Wongsrikeao et al. 2011). Oocytes with diffusely stained cytoplasmic characteristics of nonviable cells and those in which chromatin was unidentifiable or not visible were classified as “degenerated”

IVF

The matured oocytes were subjected to IVF, as described previously (Do et al., 2015). Briefly, spermatozoa from a Large White fertile boar, aged 1.5 years were frozen according to Ikeda et al. (2002) with minor modifications. The sperm-rich fraction of the ejaculate was diluted with Modena extender. After centrifugation of the extended semen, the sperm pellet was resuspended in Niwa and Sasaki freezing (NSF) extender, and then cooled to 5°C within 2 hr. Spermatozoa were then mixed with an equal volume of NSF containing 6% (v/v) glycerol and 1.48% (v/v) Equex STM (Miyazaki-kagaku, Tokyo, Japan). The sperm suspension was transferred to 0.25 ml straws, which were frozen in liquid nitrogen vapor and finally stored in liquid nitrogen until use. The straws were thawed in a water bath at 38°C for 15 s.

The frozen-thawed spermatozoa were transferred into 6 ml of porcine fertilization medium (PFM; Research Institute for the Functional Peptides Co., Yamagata, Japan) and washed by centrifuging at 500 x g for 5 min. The pelleted spermatozoa were resuspended in fertilization medium and adjusted to 5×10^6 sperms/ml. Next, COCs were transferred to the sperm-containing fertilization medium and co-incubated for 12 h at 39°C under 5% CO_2 and 5% O_2. After coincubation, the inseminated zygotes were denuded from the cumulus cells and the attached spermatozoa by mechanical pipetting.

IVC and assessment of blastocyst quality

The remaining denuded zygotes were subsequently transferred to 500 μl of porcine zygote medium-5 (PZM-5, Research Institute for the Functional Peptides Co.) in four-well dishes. Each well contained approximately 50 presumed zygotes. The zygotes were cultured continuously in vitro at 39°C in a humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2. All of the cleaved embryos were transferred into 500 μl of porcine blastocyst medium (PBM, Research Institute for the Functional Peptides Co.) 72 h after insemination, and cultured for an additional 4 days to evaluate their ability to develop to the blastocyst stage.

To evaluate the total cell number and existence of apoptosis in the blastocysts, the blastocysts were fixed on day 7 (day 0: insemination) and were

analyzed using a combined technique for simultaneous nuclear staining and terminal deoxynucleotidyl transferase nick-end labelling (TUNEL), which was described in previous chapter. Apoptotic nuclei exhibited condensed and fragmented morphology (Brison & Schultz, 1997). The apoptotic index was calculated by dividing the number of cells containing apoptotic nuclei (labeled by TUNEL) by the total number of cells.

Experimental design

To evaluate the effects of CGA supplementation during IVM culture on the IVM of oocytes and development of porcine zygotes with or without electro-stimulation treatment, the COCs were cultured in maturation medium supplemented with 10, 50, and 100 μM CGA (Sigma-Aldrich). As a control, COCs were cultured in maturation medium without CGA. After maturation culture for 44 h, the COCs were fertilized in vitro and then cultured in vitro as described above. Some zygotes received electro-stimulation treatment after IVF as described below. Electro-stimulation was performed 13 hr after the initiation of IVF as described previously (Tanihara et al., 2016). Briefly, an electrode (LF501PT1-20; BEX, Tokyo, Japan) was connected to a CUY21EDIT II electroporator (BEX) and placed under a stereoscopic microscope. The putative zygotes (approximately 30–40 zygotes) were washed with Opti-MEM I solution (Gibco Life Technologies, Carlsbad, CA, USA) and placed in a line in the

electrode gap that was in a chamber slide filled with 10 μl of Opti-MEM I solution. The putative zygotes were electroporated by electro-stimulation at 30 V/mm with five 1 msec unipolar pulses. After electro-stimulation treatment, the zygotes were cultured for 7 days as described above.

Statistical analysis

Statistical significance was inferred from analysis of variance (ANOVA) tests followed by Fisher's protected least significant difference (PLSD) tests using STATVIEW (Abacus Concepts, Inc., Berkeley, CA, USA). Percentage data were subjected to arcsin transformation before statistical analysis. Differences with a probability value (p) of 0.05 or less were regarded as significant.

RESULTS

In vitro development and quality of porcine embryos without electro-stimulation derived from oocytes matured with various concentrations of CGA.

The effects of CGA concentrations on the oocyte maturation rate, the blastocyst formation rate, the apoptotic nucleus indices and total cell number without electro-stimulation treatment are shown in Table 3.1. Supplementation of CGA at 50 μM during IVM culture significantly increased the maturation rate of oocytes and the total cell number in blastocysts compared to those of the oocytes without CGA treatment ($p < 0.05$). Porcine blastocysts derived from oocytes treated with 50 μM CGA showed significantly lower apoptotic nucleus indices than those of blastocysts without CGA treatment ($p < 0.05$). However, an increase of CGA to 100 μM did not improve the developmental competence of oocytes. There were no differences in blastocyst formation rate between the four groups.

In vitro development and quality of electro-stimulated porcine embryos derived from oocytes matured with various concentrations of CGA.

The effects of CGA supplementation during IVM culture on the development and quality of embryos electro-stimulated after IVF are shown in Table 3.2. The blastocyst formation rate in the 50 μM CGA treatment group was

significantly higher than that in the CGA-untreated and the 10 μM CGA-treated group ($p < 0.05$). Supplementation of CGA at 50 μM during IVM culture also significantly decreased the apoptotic nucleus index of blastocysts compared to that of the untreated group ($p < 0.05$). No differences in total cell number in blastocysts were found among the groups.

DISCUSSION

Electroporation, a method to produce transgenic animals, has recently been developed since microinjection is more time-consuming and skill-demanding. However, direct contact between the electrodes and the embryos placed in a small volume of liquid still currently yields the main harmful side effect, which is apoptosis that triggers cell death (Nuccitelli et al. 2010) due to caspase activation (Beebe et al. 2003), chromosomal condensation (Nuccitelli et al. 2006), or DNA fragmentation (Nuccitelli et al. 2009). To prevent unfavorable side effects in electroporated embryos, the electroporation parameters of pulse number, amplitude and frequency as well as the embryo culture system should be optimized to accomplish high-impact goals. We therefore investigated a satisfactory embryo culture system in this study as a predictive model of porcine transgenesis using electroporation.

In vitro environments for embryo culture systems contain a higher oxygen concentration than those of in vivo environments within the lumen of the female

reproductive tract (Mastroianni and Jones 1965). High oxygen concentration is predominantly associated with an increase in the production of ROS and leads to oxidative stress in the oocytes and embryos (Agarwal et al. 2003, Agarwal 2006). The balance between oxygen factors and antioxidants in the culture medium is therefore very important to drive the success of the IVP system. Administration of CGA, a dietary polyphenol shown to have antioxidant activity (Rice-Evans et al. 1996; Sato et al. 2011), to the maturation medium has recently shown the potential to improve porcine IVP (Nguyen et al. 2017). To confirm whether the CGA has any adverse effect on the developmental competence of the embryo, we examined the quality and development of porcine embryos without electro-stimulation by CGA supplementation with different concentrations during IVM. Our results showed that there were no adverse effects of CGA supplementation at a concentration of 50 μM on the development of embryos. CGA had a beneficial effect on the maturation rate of oocytes, the total cell number, and the apoptotic nucleus indices of blastocysts

Multiple pulses can cause cell injury and apoptosis, a part of cellular stress, resulting in the release of ROS from the mitochondria to the cytoplasm (Kuznetsov et al. 2011, Shil et al. 2005) and oxidative stress on the embryos. Next, we evaluated the development and quality of electroporated porcine embryos derived from oocytes matured with various concentrations of CGA. The results indicated that addition of CGA at a 50 μM concentration also shows potential for positive effects on the blastocyst formation rate and the apoptotic

nucleus indices of blastocysts. Our results are in agreement with those of Nguyen et al. (2017), who reported that supplementation with 50 μM CGA is advantageous to the porcine IVP system. When porcine zygotes from oocytes matured with or without 50 μM CGA were electroporated with Cas9 messenger RNA and single guide RNA targeting sites in pancreatic duodenal homeobox-1 (Pdx1) gene (Wu et al. 2017), the proportion (89.3%, 25/28) of blastocysts with a mutated sequence in the CGA treated group was similar with that (88.2%, 30/34) in the untreated group (data not shown). These observations indicate that CGA supplementation during maturation culture may increase the number of embryos with insertions or deletions (indels) in the targeted gene by electropolation.

Hydrogen peroxide (H_2O_2), a member of the ROS family, is remarkably accumulated under abiotic stress conditions and can readily diffuse through cell membranes (Nguyen et al. 2017, You and Chan 2015). An increase in the number of apoptotic embryos with DNA fragmentation appears to be related to increasing H_2O_2 concentrations (Lee and Yeung 2006, Yang et al. 1998). In the present study, the improvement of porcine embryo development and quality by CGA supplementation during IVM culture might be explained through its antioxidant activity. The exposure to CGA during IVM culture may potentially decrease H_2O_2-induced apoptotic cell death by up-regulating anti-apoptotic proteins and preventing H_2O_2-induced caspase activation (Kim et al. 2012, Rebai et al. 2017), leading to the depletion of ROS in the electroporated porcine embryos. The oocytes and embryos are then protected from oxidative stress that

could be induced by either a high oxygen concentration under IVC conditions or electro-stimulation treatment.

In conclusion, our findings support that the administration of CGA may help to improve the developmental competence and quality of porcine IVP embryos, especially electro-stimulation treated porcine zygotes. To ascertain the best model for porcine transgenesis, additional investigation of pulse number, amplitude and frequency electro-stimulation parameters is further required.

Table.3.1. Effects of chlorogenic acid (CGA) supplementation during in vitro maturation on the development and quality of embryos[*]

Concentration of CGA (μM)	Number of examined oocytes	Number (%) of matured oocytes	Number of examined oocytes	Number (%) developed to blastocysts	Total cell number in blastocyst	Apoptotic nucleus index **
0	73	52 (64.7 ± 5.2)[a]	208	6 (3.0 ± 0.7)	32.3 ± 3.6[a]	16.0 ± 3.7[a]
10	83	65 (75.9 ± 3.9)[a,b]	212	7 (3.3 ± 1.8)	47.1 ± 8.9[a,b]	14.2 ± 1.8[a,b]
50	84	68 (80.7 ± 6.1)[b]	218	13 (6.1 ± 1.3)	56.0 ± 7.6[b]	10.2 ± 1.4[b]
100	82	64 (77.4 ± 4.5)[a,b]	212	13 (6.1 ± 2.8)	45.0 ± 6.1[a,b]	10.0 ± 0.9[b]

[*]Four replicate trials were carried out. Data are expressed as the mean ± SEM.

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

[a,b]Values with different superscripts in the same column are significantly different ($p < 0.05$).

Table.3.2. Effects of chlorogenic acid (CGA) supplementation during in vitro maturation on the development and quality of embryos electroporated after in vitro fertilization*

Concentration of CGA (μM)	Number of examined oocytes	Number (%) developed to blastocysts	Total cell number in blastocyst	Apoptotic nucleus index **
0	214	3 (1.4 ± 1.0)[a]	47.0 ± 2.1	13.0 ± 3.0[a]
10	224	4 (1.8 ± 0.9)[a]	35.3 ± 3.8	12.1 ± 1.3[a,b]
50	210	10 (4.7 ± 0.5)[b]	41.2 ± 4.2	7.6 ± 0.8[b]
100	217	7 (3.2 ± 0.8)[a,b]	39.7 ± 7.1	10.6 ± 1.9[a,b]

*Six replicate trials were carried out. Data are expressed as the mean ± SEM. Electro-stimulation was performed by five 1 msec pulses at 30V.

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

[a,b]Values with different superscripts in the same column are significantly different ($p < 0.05$).

GENERAL DISCUSSION AND CONCLUSION

The history of IVP system in pigs has been recognized for decades since the first IVM porcine oocytes were fertilized (Motlik and Fulka 1974). However, in comparison with those of other mammalian species such as cattle or mice, its efficiency is still low. Whereas, the demand for a huge quantity and excellent quality of in vitro produced pig embryo is dramatically increasing recently because of the development in the field of assisted reproductive technologies, gene engineering and embryonic stem cells. Either matured oocytes, zygotes or blastocysts are considered as important materials, critically used not only for basic sciences such as physiology and reproduction, but also for advance biotechnology and biomedical research. Furthermore, pigs have shown the physiological similarities to humans leading to the rise in an economic interest in swine biotechnologies used for biomedical and swine industries. The interest has created an increased desire for new technologies as well as an urge for implementation of the existing ones. At this point, the IVP of swine embryos is interesting to researchers. These animals can be used as human bio-models for creating genetically modified animals as potential donors of tissues and organs for further xenotransplantation (Gil et al. 2010).

Several antioxidants have shown the ability to inhibit oxidative stress induced under in vitro conditions where the oxygen concentration is found to be higher than that within female reproductive tract (Mastroianni and Jones 1965). There are two basic categories of antioxidants, natural and synthetic, the natural

products rich in antioxidants are of great importance for scientists. There is increasing interest in phenolic compounds and their antioxidant activity among consumers and the scientific community in the past decade because of their relatively low toxicity and high bioactivities (Kaur Kala et al. 2016). One of the powerful phenolic compound is chlorogenic acid that usually presenting in coffee, leaves and fruits of several kinds of plants (Cho et al. 2010, Mahmood et al. 2012). CGA is also referred to as 5-O-caffeoylquinic acid, which is an ester of caffeic acid and quinic acid (Gonthier et al. 2006).

In the first study (Chapter 1), we firstly examined the effects of CGA supplementation during IVM on the maturation, fertilization and development of porcine oocytes then investigate its antioxidant ability by exposure oocytes into hydrogen peroxide (H_2O_2). The data indicated that, at concentration of 50 μM, CGA effectively improved the rate of maturation, fertilization and development to blastocyst stage as well as protected oocytes from oxidative stress induced by H_2O_2. As a conjugate of caffeic acid, CGA has been confirmed to be able to quench a ROS (Gulcin 2006). In this study, CGA has shown a similar effect with caffeic acid in supporting porcine oocytes IVM and their development after IVF. Furthermore, heat stress has been considered as a crucial factor that can disrupt reproductive process by reducing intracellular concentration of the antioxidant glutathione in embryos (Hansen and Arechiga 1999). Exposure to heat stress has detrimental effects on oocyte cytoskeletal organization and meiotic and development competence (Ju et al. 2004, Barati et al. 2008, Tong et al. 2004).

Thus, in the second study (Chapter 2), we have investigated the effects of CGA supplementation during IVM on the ability of maturation and their development after parthenogenesis activation when oocytes exposed to elevated temperature. The supplementation of CGA at concentration of 50 μM during IVM has potential to restore maturation rate and protect oocytes from apoptosis process. Moreover, the present of CGA in maturation medium could improve the quality of blastocyst after parthenogenesis activation.

The use of animal models has been a valuable tool for using in both basic science and other advanced studies. The similarity in size and physiology, organ development and disease progression make the swine an ideal research model for humans (Bolton 1997, Schook et al. 2005) including translational biomedical research. There have been many pig models with genetically modifications via SCNT (Lai et al. 2002, Lai et al. 2006, Rogers et al. 2008, Suzuki et al. 2012), microinjection (Uchida et al. 2001) and sperm-mediated gene transfer (SMGT) (Lavitrano et al. 2003). Since most of transgenic pigs have been produced by random integration, the necessity for more exact gene-mutated models using recombinase based conditional gene expression like mice has thus been raised. Currently, advanced genome-editing technologies enable us to generate specific gene-deleted and -inserted pig models. In vitro electroporation mediated gene delivery using IVF zygotes has been reported as an efficient method because it does not require advanced skills and reduce considerable time (Kaneko et al. 2014, Hashimoto and Takemoto 2015; Sato et al. 2016). Moreover,

electroporation method has the potential to facilitate the large-scale production of mutant pigs and will be applicable to the genetic modification of other mammals, in addition to pigs, it will undoubtedly contribute to the advancement of biomedical and agricultural research (Tanihara et al. 2016). In the third study (Chapter 3), we therefore evaluated the protective effects of various concentrations of CGA on the developmental competence of electro-stimulation treated porcine embryos derived from oocytes matured in vitro. In agreement with first and and second studies, the supplementation of CGA at concentration of 50 μM has also shown the positive effects on the blastocyst formation rate and the apoptotic nucleus index.

Oxidative stress certainly harms 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. Moreover, it has been suggested that electroporation under conditions compatible with cell survival induces lipid hydroperoxide formation in the cell membranes (Maccarrone et al. 1995). Singlet oxygen is another product of electroporation-induced lipid peroxidation (Maccarrone et al. 1995). Therefore, the use of electroporation may induce cellular stress that results in the accumulation of ROS in embryonic cell cytoplasm (Maccarrone et al. 1995, Shil et al. 2005). In the third study (Chapter 3), we have evaluated the apoptotic index of the oocytes and blastocysts to investigate antioxidant effects of CGA. The results showed that the exposure to

CGA during IVM was effectively protect porcine oocytes and embryos from apoptotic process which could be generating under oxidative stress during IVC conditions or electro-stimulation treatment.

CGA has been known as a common phenolic antioxidant exhibits several effects on human health. To the best of our knowledge, the present study was the first report concerning about effects of CGA supplementation on in vitro development and quality of porcine oocytes/ embryos. Taken together, our results suggested that the supplementation of 50 μM CGA during IVM has beneficial effects on embryonic development and quality of porcine embryos with or without electro-stimulation treatment.

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