ABSTRACT
In this study, we aimed to test whether embryonic development of blastocysts following injection of mouse induced pluripotent stem (iPS) cells can be improved by the supplementation of antioxidants during in vitro maturation. For this, we evaluated the effects of chlorogenic acid (CGA) and insulin–transferrin–selenium (ITS) supplementation either alone or in combination during oocyte maturation on the quality of porcine blastocysts and the embryonic development after injection of mouse iPS cells. Our results demonstrate that, prior to injection of mouse iPS cells, CGA or ITS supplementation either alone or in combination had no effects on the rates of oocyte maturation, oocyte DNA fragmentation, fertilization, and blastocyst formation. However, the combination of CGA and ITS significantly increased the total cell number in blastocysts compared with the control group (P < 0.05). When mouse iPS cells were injected into porcine blastocysts, CGA supplementation either alone or in combination with ITS had a beneficial effect on the development of interspecies chimera blastocysts. In conclusion, our results indicate that supplementation of maturation medium with a combination of CGA and ITS enhances the ability of porcine oocytes to develop interspecies chimera blastocysts following iPS cell injection.

Introduction

Generation of fully pluripotent stem cell-derived tissues or organs in a chimeric animal can be produced by injecting wild-type pluripotent stem cells into blastocysts derived from a genetically deficient animal (Wu et al. 2017; Suchy and Nakachi 2018). Following blastocyst injection, chimeric cell survival and proliferation during embryogenesis are influenced by several factors including variations in systemic contribution of the donor cell cycle and cell culture conditions (Masaki et al. 2016; Suchy and Nakachi 2018). In the production of chimeric animals, however, obtaining high-quality blastocysts is the most critical factor for generating live chimeric animals after the transfer of embryos with the injection of pluripotent stem cells (PSCs).

In pigs, the development and quality of embryos produced by the current in vitro production system involving in vitro oocyte maturation, fertilization, and embryo culture are lower than those of in vivo-derived embryos (Kikuchi et al. 2008). In vitro maturation (IVM) of oocytes is considered to be the most crucial step for in vitro embryo production (IVP) (Jeong et al. 2008). Despite the technical developments in IVM over the years, a major limitation of the in vitro culture system is the imbalance between oxygen concentration and antioxidant protection, resulting in an increase in the generation of reactive oxygen species (ROS), which in turn increase the oxidative stress of oocytes (Agarwal et al. 2003; Agarwal et al. 2006). The supplementation of chlorogenic acid (CGA), known to have free radical scavenging properties (Gonthier et al. 2006; Castro et al. 2018), to the maturation medium has shown potential in improving maturation, fertilization and developmental competence of porcine oocytes (Nguyen et al. 2017; Nguyen et al. 2018). In addition, insulin–transferrin–selenium (ITS) has been routinely used in several IVM systems to promote the growth of oocytes retrieved from sheep (Mondal et al. 2015) and mouse (De La Fuente et al. 1999). As a polypeptide hormone, insulin promotes the uptake of glucose and amino acids and thus improves the developmental competence of porcine oocytes (Kikuchi et al. 1999; Kim et al. 2005). Transferrin has a prominent growth-promoting activity that exerts a supportive effect on in vitro embryo development through the binding of iron (Fe³⁺), making it less toxic (Natsuyama et al. 1993). Selenium functions as an essential component of glutathione peroxidase, which protects cells and tissue from free radicals and oxidative stress (Michiels et al. 2001).
Therefore, the use of CGA and ITS either alone or in combination may not only prevent the adverse effects of oxidative stress during IVM but also improve the quality of the resulting blastocysts.

The present study aimed to examine the effects of CGA and ITS supplementation into the maturation medium on the quality of porcine blastocysts and the development of blastocysts after mouse induced pluripotent stem (iPS) cell injection to produce interspecies chimera.

Materials and methods

General

All procedures were approved by the Animal Research Committee of Tokushima University.

Oocyte collection and in vitro maturation

In vitro maturation was performed according to methods described by Namula et al. (2013) with minor modifications. Briefly, porcine ovaries were obtained from approximately 6-month-old gilts at a local slaughterhouse and were transported within 1 h to the laboratory in physiological saline at 30°C. The follicles on the ovarian surface were sliced using a surgical blade in a sterilized dish under a stereomicroscope and the cumulus oocyte complexes (COCs) were collected. Approximately 50 COCs were then cultured in 500 μL maturation medium for 22 h in 4-well dishes (Nunc A/S, Roskilde, Denmark) covered with mineral oil (Sigma-Aldrich, St. Louis, MO, U.S.A.). Maturation medium consisting of tissue culture salts (TCM 199; Thermo Fisher Scientific, Waltham, MA, U.S.A.) was supplemented with 10% (v/v) porcine follicular fluid, 50 μM sodium pyruvate (Sigma-Aldrich), 2 mg/mL D-sorbitol (Wako Pure Chemical Industries Ltd., Osaka, Japan), 0.6 mM cysteine (Sigma-Aldrich), 10 IU/mL equine chorionic gonadotropin (Asuka Pharmaceutical, Tokyo, Japan), 10 IU/mL human chorionic gonadotropin (Nippon Zenyaku Kogyo, Fukushima, Japan), and 50 μg/mL gentamicin (Sigma-Aldrich). Subsequently, the COCs were transferred into maturation medium without hormone supplementation and cultured for an additional 22 h. COCs were incubated at 39°C in a humidified incubator containing 5% CO₂ in air.

Assessment of meiotic status and DNA fragmentation

To assess the meiotic status and DNA fragmentation of oocytes following IVM, some oocytes were fixed at 44 h of culture and analysed using a technique for simultaneous nuclear staining with DAPI (Nacalai Tesque Inc., Kyoto, Japan) and terminal deoxynucleotidyl transferase nick-end labelling (TUNEL) according to the method described by Do et al. (2015). Based on their chromatin configuration, they were classified as ‘germinal vesicle’, ‘condensed chromatin’, ‘metaphase I’, or ‘metaphase II’. Oocytes with diffusely stained cytoplasm characteristic of nonviable cells and those in which chromatin was undetectable or not visible were classified as ‘degenerated’ and excluded from DNA damage analysis.

In vitro fertilization and assessment

In vitro fertilization (IVF) was performed according to the method described by Nguyen et al. (2017). Freeze-thawed spermatozoa obtained from a boar were transferred into 6 mL of porcine fertilization medium (PFM; Research Institute for the Functional Peptides Co., Yamagata, Japan) and washed by centrifugation at 500 × g for 5 min. The pelleted spermatozoa were re-suspended in PFM and adjusted to a cell density of 1 × 10⁶ cells/mL. Oocytes were then transferred to the sperm-containing PFM and co-incubated for 5 h at 39°C, in a humidified incubator containing 5% CO₂, 5% O₂ and 90% N₂.

To assess oocyte fertilization, some presumptive zygotes were mounted on glass slides 10 h after insemination 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 phase contrast microscope. Oocytes containing both female and male pronuclei were considered to be fertilized and were categorized as monospermic or polyspermic depending on the number of swollen sperm heads and/or pronuclei in the cytoplasm (Do et al. 2015). The proportion of monospermic fertilization was calculated by dividing the number of monospermic fertilized oocytes by the total number of fertilized oocytes.

In vitro culture and assessment of blastocyst quality

After the co-incubation, the inseminated oocytes were denuded from the cumulus cells and attached spermatozoa by mechanical pipetting. Approximately 50 denuded presumptive zygotes were subsequently cultured in 500 μL PZM-5 (Research Institute for the Functional Peptides Co.) in 4-well dishes. The presumptive zygotes were cultured in vitro at 39°C in a humidified incubator with 5% CO₂, 5% O₂ and 90% N₂. The cleaved embryos were transferred into 500 μL porcine blastocyst medium (PBM; Research Institute for the Functional Peptides Co.) 72 h after insemination, and were cultured for an additional 4 days to evaluate their ability to develop to the blastocyst stage. All embryos were fixed on Day 7 (Day 0 = insemination) and stained with Hoechst 33342 (Sigma-Aldrich). Their cell numbers were counted to assess the quality and development stage of the embryos.

Preparation of mouse iPS cells

The mouse iPS line-iPS-Stm-FB/gfp-99-1 (Aoi et al. 2008) which expresses a green fluorescent protein (GFP) was provided by RIKEN Bio Resource Center, Japan. iPS cells were cultured in iPS cell culture medium (ESGRO complete PLUS clonal grade medium, Millipore, Billerica, MA) without feeder cells. The cells were passaged every 3–4 days.

Injection of iPS cells and embryos culture

Blastocysts with an obvious blastocoel were collected on Day 6 and were used for iPS cells injections. The injection was performed in a mixture medium (20 μL drop) comprising of iPS cell culture medium and PBM (1:1, v/v) covered by mineral oil. After the blastocysts were immobilized with a holding
piptette, the inner cell mass (ICM) was placed at the 6, 9 or 12 o'clock position (Figure 1A) and the injection pipette was inserted into the blastocoel through the zona pellucida at the 3 o'clock position. The injection pipette containing about 10 iPS cells was put in the blastocoel of blastocysts. After injection of iPS cells (Figure 1B), the blastocysts were cultured in the mixture medium containing 5% FBS for another 24 h in 4-well dishes. After 24 h blastocysts that regained their original shape with a fully re-expanded blastocoel and those that had hatched from zona pellucida were classified as ‘re-expanded blastocyst’ (Figure 1C) and ‘hatched blastocyst’ (Figure 1D), respectively. At the end of the culture period, the GFP signal was observed using an inverted fluorescence microscope (ECLIPSE Ti-U, Nikon, Tokyo, Japan).

Experimental design

In the first experiment, we evaluated the effects of CGA and ITS supplementation during IVM culture (44 h) on the in vivo maturation (146–152 oocytes per treatment), fertilization (139–152 oocytes per treatment), and development (199–230 oocytes per treatment) of porcine oocytes. COCs were matured in maturation medium supplemented with 50 μM CGA (C3878, Sigma-Aldrich) dissolved in dimethyl sulphoxide or ITS (1.0 mg/mL insulin, 0.55 mg/mL transferrin, and 0.5 μg/mL selenium, I3146, Sigma-Aldrich), either alone or in combination. As a control, COCs were cultured in maturation medium containing 50 μM β-mercaptoethanol (Wako Pure Chemical Industries Ltd.) instead of ITS and CGA. All of the experiments were repeated four to five times.

In the second experiment, we evaluated the effects of ITS and CGA supplementation during IVM culture on the development of blastocysts (54–64 embryos per treatment) following injection of mouse iPS cells. Mouse iPS cells were injected into the blastocoel of blastocysts collected on Day 6, and their development was examined as described in ‘injection of iPS cells and embryos culture’ section. Blastocysts from COCs matured in the maturation medium containing β-mercaptoethanol instead of CGA and ITS served as the control group. All of the experiments were repeated four times.

Statistical analysis

Experiments were repeated four to five times. 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 prior to statistical analysis. Differences with a probability value ($P < 0.05$) were considered statistically significant.

Results

The effects of CGA and ITS supplementation on in vitro maturation, fertilization, and development of porcine oocytes during IVM culture were examined. The supplementation of ITS and CGA either alone or in combination into the maturation medium had no beneficial effects on the rates of maturation and DNA fragmentation of oocytes compared to the control group (Figure 2A and B; 88.5%–92.1% and 1.3%–3.1%, respectively). Moreover, the rates of total fertilization and monospermic fertilization of oocytes did not differ among the supplementation groups (Figure 2C and D; 62.2%–69.8% and 91.2%–94.0%, respectively). A significantly higher total cell number in blastocysts was found in the embryos derived from oocytes matured with the combination of CGA and ITS when compared to control and ITS groups (Figure 2F; 51.5 vs 42.0 and 44.2, $P < 0.05$). However, no significant differences in the rates of blastocyst formation were observed among the groups (Figure 2E; 24.2%–29.1%).

When the effects of CGA and ITS supplementation during in vitro maturation on the development of embryos following injection of mouse iPS cells into blastocysts were examined, there was no significant difference in the rates of re-expanded blastocysts among the treatment groups (Table 1). However, the rates of hatched blastocysts derived from oocytes matured with CGA alone and in combination with ITS were significantly higher than those of the other groups.

Discussion

There have been many improvements in the in vitro culture system to enable production of high-quality oocytes and embryos. However, the ability of embryos to develop further in vitro remains insufficient when compared to the in vivo system (Kikuchi et al. 2008). In the most porcine IVF systems, β-mercaptoethanol is widely used as an antioxidant in porcine IVM cultures to generate high-quality embryos (Funahashi 2005; Choe et al. 2010). Therefore, the control group used in the present study contained β-mercaptoethanol instead of CGA and ITS in the maturation medium. Our results show that the combined supplementation of CGA and ITS during IVM culture resulted in a higher total cell number.
Figure 2. Effects of chlorogenic acid (CGA) and ITS supplementation during in vitro maturation on the proportions of oocytes reaching metaphase II (A) and oocytes with DNA fragmented nuclei (B), total fertilization (C), monospermic fertilization (D), blastocyst formation (E) and the total cell number of blastocysts (F). Oocytes matured in the maturation medium containing β-mercaptoethanol but not CGA and ITS served as the control group. All oocytes matured with or without CGA and ITS were used to estimate the proportions of oocytes reaching metaphase II (MII) and having nuclei with fragmented DNA (146–152 oocytes per treatment), and of fertilization (139–152 oocytes per treatment) and embryo development (199–230 oocytes per treatment). All the experiments were repeated four to five times. Each bar represents the mean value ± SEM. Bars with different letters differ significantly (P < 0.05).
in blastocysts compared with β-mercaptoethanol supplementation. These results are supported by our previous finding that CGA supplementation at a concentration of 50 µM during the IVM process is beneficial for porcine IVP system owing to CGA protecting the oocytes from DNA damage caused by oxidative stress (Nguyen et al. 2017). The present study also reveals that ITS supplementation in maturation medium did not improve the rates of blastocyst formation and total cell number in blastocysts. Our results are in agreement with those of Shamsuddin et al. (1994) who supplemented ITS in the maturation medium of bovine oocytes and observed that the percentage of cleavage and blastocyst formation was not different from that of the control group. However, interestingly, this study suggests that there is a synergistic effect on the total cell number in blastocysts when a combination of CGA and ITS was used. This synergistic effect may in part be due to enhanced antioxidant defenses by selenium in ITS that has antioxidant activity (Michiels et al. 1994). In a previous study, ITS supplementation during IVM has been shown to improve the developmental competence of porcine oocytes (Jeong et al. 2008), which is not consistent with the findings of the present study. Moreover, we found that the combined supplementation of CGA and ITS during IVM culture did not improve the oocyte maturation, oocyte quality, fertilization, and blastocyst formation compared with β-mercaptoethanol supplementation. These results indicate that no apparent effects of the combination of CGA and ITS were observed because all treatment groups contained antioxidants such as CGA, ITS, and β-mercaptoethanol. Therefore, further studies will be necessary to clarify the effect of the combination of CGA and ITS by use of IVM medium without any antioxidant supplementation as control group.

To the best of our knowledge, this is the first report showing the beneficial effects of supplementation of CGA in combination with ITS on the hatched rate of interspecies chimera blastocysts. Following mouse iPS cell injection, the blastocysts shrank instantly due to the creation of holes in zona pellucida, resulting in the loss of blastocoel fluid and decrease in blastocyst developmental competence. The blastocoel fluid plays a crucial role in direct to support cellular processing and blastocyst development (Pedersen and Spindle 1980; Jensen et al. 2014). However, the interspecies chimera blastocysts could further re-expand, develop, and completely hatch after 24 h of culture. The rate of embryos hatched from zona pellucida has been reported as an indicator of adequacy of culture conditions (Fléchon and Renard 1978). In the present study, our results showed that the rate of hatched blastocysts derived from oocytes treated with CGA either alone or in combination with ITS was higher than that in the other groups. This suggests that the combination of CGA and ITS may have a positive effect on the in vitro development of interspecies chimera blastocysts because of the improvement in blastocyst quality. Moreover, considering the improvement of embryonic development by the combination of CGA and ITS, further investigations are needed to clarify whether the supplementation of CGA in combination with ITS during the culture of interspecies chimera blastocysts may increase the rate of hatched blastocysts.

In conclusion, although the CGA and ITS supplementation of porcine oocyte maturation medium did not positively affect the rates of oocyte maturation and fertilization, the total cell number in blastocysts was improved by the combined supplementation of CGA and ITS. Moreover, the development of blastocysts after mouse iPS cells injection also improved when CGA was used either alone or in combination with ITS. Thus, we conclude that the combination of CGA and ITS supplementation in the maturation medium could be beneficial to the porcine IVP system for producing interspecies chimera blastocysts.

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Disclosure statement
No potential conflict of interest was reported by the author(s).

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