Effects of chlorogenic acid (CGA) supplementation during *in vitro* maturation culture on the development and quality of porcine embryos with electroporation treatment after *in vitro* fertilization

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Running title:
EFFECT OF CGA ON ELECTROPLATED EMBRYOS

ABSTRACT

Electroporation is the technique of choice to introduce an exogenous gene into embryos for transgenic animal production. Although this technique is practical and effective, embryonic damage caused by electroporation treatment remains a major problem. This study was conducted to evaluate the optimal culture system for electroporation-treated porcine embryos by supplementation of chlorogenic acid (CGA), a potent antioxidant, during *in vitro* oocyte maturation. The oocytes were treated with various concentrations of CGA (0, 10, 50, and 100 μM/L) through the duration of maturation for 44 h. The treated oocytes were then fertilized, electroporated at 30 V/mm with five 1-msec unipolar pulses, and subsequently cultured *in vitro* until development into the blastocyst stage. Without electroporation, the treatment with 50 μM/L 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 electroporated after *in vitro* fertilization, the treatment with 50 μM CGA supplementation significantly improved the rate of oocytes that developed into blastocysts and 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 electroporation-treated embryos.

Keywords: electroporation, chlorogenic acid, antioxidant, oxidative stress, embryo

53 INTRODUCTION

54

55 A transgenic animal model is a powerful tool for developing a more detailed
56 understanding of gene regulation and function in biological systems (Boverhof *et al.*
57 2011). This technology introduces an exogenous gene into the embryonic cells' genome,
58 where it is expressed and inherited by offspring. Some techniques, such as microinjection,
59 somatic cell nuclear transfer (SCNT), and sperm-mediated gene transfer (SMGT), are
60 available for the production of transgenic animals, and each is of value in certain
61 circumstances. Over the last three decades, microinjection has been widely accepted as
62 the gold standard to transfer the exogenous gene into the animal genome at random sites
63 (Meyer *et al.* 2010; Garrels *et al.* 2011; Wongsrikeao *et al.* 2011; Yang *et al.* 2013; Li *et*
64 *al.* 2014; Proudfoot *et al.* 2015). Pigs *(Sus scrofa)* have been recognized as an important
65 model organism for several biomedical types of research including animal transgenesis
66 since they exhibit anatomical and physiological commonalities almost identical to
67 humans (Ramsoondar *et al.* 2009; Samiec & Skrzyszowska 2011). Although the
68 microinjection of exogenous genes into the porcine zygotes resulted in successful
69 production of transgenic piglets with an acceptable germline transmission rate to their
70 offspring (Garrels *et al.* 2011; Ivics *et al.* 2014; Li *et al.* 2014), the involvement of skilled
71 personnel and extended periods of micromanipulation causing severe embryonic damage
72 are a major limitation of this technique (Iqbal *et al.* 2009). Electroporation has thus
73 become an alternative technique due to its ease of use and sufficient embryonic survival
74 rate (Kaneko *et al.* 2014).

75 The technique of electroporation has been primarily utilized to introduce foreign
76 DNA into a donor cell for use in producing transgenic animals by SCNT (Ross *et al.*
77 2010). It later was used successfully to create gene knockout and gene knock-in mice and
78 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 *et al.* 2014; Kaneko & Mashimo 2015). 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 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 reactive oxygen species (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.* 2003; Agarwal *et al.* 2006). Antioxidant defense 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 Akkol *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 electroporation-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.

105 MATERIALS AND METHODS

106

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

108

109 **In vitro maturation (IVM) and assessment**

110 Porcine ovaries were obtained from approximately 6-month-old gilts at a local
111 slaughterhouse and were transported within 1 h to the laboratory in physiological saline
112 at 30°C. Ovaries were placed in modified phosphate-buffered saline (m-PBS;
113 Nihonzenyaku, Fukushima, Japan) supplemented with 100 IU/mL penicillin G potassium
114 (Meiji, Tokyo, Japan) and 0.1 mg/mL streptomycin sulfate (Meiji). The follicles on the
115 ovarian surface were sliced using a surgical blade on the sterilized dish. Only cumulus-
116 oocyte complexes (COCs) with a uniformly dark-pigmented ooplasm and intact cumulus
117 cell masses were collected under a stereomicroscope. Approximately 50 COCs were then
118 cultured in 500 μL of maturation medium, consisting of 25 mM/L HEPES tissue culture
119 medium 199 with Earle's salts (TCM 199; Invitrogen Co., Carlsbad, CA, USA)
120 supplemented with 10% (v/v) porcine follicular fluid, 50 μM/L sodium pyruvate (Sigma-
121 Aldrich), 2 mg/mL D-sorbitol (Wako Pure Chemical Industries Ltd.), 10 IU/mL equine
122 chorionic gonadotropin (Kyoritu Seiyaku, Tokyo, Japan), 10 IU/mL human chorionic
123 gonadotropin (Kyoritu Seiyaku), and 50 μg/mL gentamicin (Sigma-Aldrich) for 22 h in
124 4-well dishes (Nunc A/S, Roskilde, Denmark). Subsequently, the COCs were transferred
125 into maturation medium without hormone supplementation and cultured for an additional
126 22 h. The incubation of COCs was conducted at 39°C in a humidified incubator
127 containing 5% CO_2 in air.

128 To assess the meiotic status of oocytes following IVM, some oocytes were denuded,
129 fixed, and permeabilized in Dulbecco's PBS (DPBS; Invitrogen), supplemented with
130 3.7% (w/v) paraformaldehyde and 1% (v/v) Triton X-100 (Sigma-Aldrich) at 25°C for 15

131 min. Permeabilized oocytes were then placed on glass slides and stained with 1.9 mM/L
132 bisbenzimide (Hoechst 33342; Sigma-Aldrich) before being covered with coverslips.
133 After overnight incubation at 4°C, the oocytes were examined by fluorescence
134 microscopy. Based on their chromatin configuration, they were classified as 'germinal
135 vesicle', 'germinal vesicle breakdown', 'metaphase I', or 'metaphase II' (Wongsrikeao et
136 al. 2004). Oocytes with diffusely stained cytoplasmic characteristics of nonviable cells
137 and those in which chromatin was unidentifiable or not visible were classified as
138 'degenerated.'
139
140 **In vitro fertilization**
141 The matured oocytes were subjected to *in vitro* fertilization (IVF), as described
142 previously (Do *et al.* 2015). Briefly, spermatozoa from a Large White fertile boar, aged
143 1.5 years were frozen according to described by Ikeda et al. (2002) with minor
144 modifications. The sperm-rich fraction of the ejaculate was diluted with Modena extender.
145 After centrifugation of the extended semen, the sperm pellet was resuspended in Niwa
146 and Sasaki freezing (NSF) extender, and then cooled to 5°C within 2 h. Spermatozoa were
147 then mixed with an equal volume of NSF containing 6% (v/v) glycerol and 1.48% (v/v)
148 Orvus ES paste (Miyazaki-kagaku, Tokyo, Japan). The sperm suspension was transferred
149 to 0.25-mL straws, which were frozen in liquid nitrogen vapor and finally stored in liquid
150 nitrogen until use. The straw was thawed in a water bath at 38°C for 15 sec.
151 The frozen-thawed spermatozoa were transferred into 6 mL of fertilization medium
152 (PFM; Research Institute for the Functional Peptides Co., Yamagata, Japan) and washed
153 by centrifuging at 500 × *g* for 5 min. The pelleted spermatozoa were resuspended in
154 fertilization medium and adjusted to 5×10^6 cells/mL. Next, COCs were transferred to
155 the sperm-containing fertilization medium and co-incubated for 12 h at 39°C under 5%
156 CO_2 and 5% O_2. After co-incubation, the inseminated zygotes were denuded from the

157 cumulus cells and the attached spermatozoa by mechanical pipetting.
158
159 **In vitro culture and assessment of blastocyst quality**
160 The remaining denuded zygotes were subsequently transferred to 500 μL of PZM-5
161 (Research Institute for the Functional Peptides Co.) in 4-well dishes. Each well contained
162 approximately 50 presumed zygotes. The zygotes were cultured continuously *in vitro* at
163 39°C in a humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2. All of the
164 cleaved embryos were transferred into 500 μL of PBM (Research Institute for the
165 Functional Peptides Co.) 72 h after insemination, and cultured for an additional 4 days to
166 evaluate their ability to develop to the blastocyst stage.
167 To evaluate the total cell number and existence of apoptosis in the blastocysts, the
168 blastocysts were fixed on day 7 (day 0; insemination) and were analyzed using a
169 combined technique for simultaneous nuclear staining and terminal deoxynucleotidyl
170 transferase nick-end labelling (TUNEL), which was modified from previously described
171 procedures (Otoi *et al.* 1999). Briefly, blastocysts were fixed overnight at 4°C in 3.7%
172 (w/v) paraformaldehyde diluted in PBS. After fixation, the blastocysts were
173 permeabilized in PBS containing 0.1% (v/v) Triton-X100 for 40 min. The blastocysts
174 were subsequently incubated overnight at 4°C in PBS containing 10 mg/mL bovine serum
175 albumin (blocking solution) and then incubated in fluorescein-conjugated 2-deoxyuridine
176 5-triphosphate and terminal deoxynucleotidyl transferase (TUNEL reagent; Roche
177 Diagnostics Co., Tokyo, Japan) for 1 h at 38.5°C. After TUNEL staining, the embryos
178 were counterstained with 1 μg/mL DAPI (Invitrogen Co., Carlsbad, CA, USA) for 10 min
179 and then treated with an anti-bleaching solution (Slow-Fade; Molecular Probes Inc.,
180 Eugene, OR, USA), mounted on glass slides and sealed with clear nail polish. Labelled
181 blastocysts were examined using an epifluorescence microscope (Eclipse 80i, Nikon,
182 Tokyo, Japan). Apoptotic nuclei exhibited condensed and fragmented morphology

183 (Brison & Schultz 1997). The apoptotic index was calculated by dividing the number
184 of cells containing apoptotic nuclei (labeled by TUNEL) by the total number of cells.
185
186 **Experimental design**
187 To evaluate the effects of CGA supplementation during IVM culture on the *in vitro*
188 maturation (IVM) of oocytes and development of porcine zygotes with or without
189 electroporation treatment, the COCs were cultured in maturation medium supplemented
190 with 10, 50, and 100 μM/L CGA (Sigma-Aldrich). As a control, COCs were cultured in
191 maturation medium without CGA. After maturation culture for 44 h, the COCs were
192 fertilized *in vitro* and then cultured *in vitro* as described above. Some zygotes received
193 electroporation treatment after IVF as described below.
194 Electroporation was performed 13 h after the initiation of IVF as described
195 previously (Tanihara *et al.* 2016). Briefly, an electrode (LF501PT1-20; BEX, Tokyo,
196 Japan) was connected to a CUY21EDIT II electroporator (BEX) and placed under a
197 stereoscopic microscope. The putative zygotes (approximately 30 – 40 zygotes) were
198 washed with Opti-MEM I solution (Gibco Life Technologies, Carlsbad, CA, USA) and
199 placed in a line in the electrode gap that was in a chamber slide filled with 10 μl of Opti-
200 MEM I solution. The putative zygotes were electroporated by electroporation at 30 V/mm
201 with five 1-msec unipolar pulses. After electroporation treatment, the zygotes were
202 cultured for 7 days as described above.
203
204 **Statistical analysis**
205 Statistical significance was inferred from analysis of variance (ANOVA) tests
206 followed by Fisher's protected least significant difference (PLSD) tests using STATVIEW
207 (Abacus Concepts, Inc., Berkeley, CA, USA). Percentage data were subjected to arcsin
208 transformation before statistical analysis. Differences with a probability value (P) of 0.05

209 or less were regarded as significant.

210

211 RESULTS

212

213 **In vitro development and quality of porcine embryos without electroporation**
214 **derived from oocytes matured with various concentrations of CGA**

215 The effects of CGA concentrations on the oocyte maturation rate, the blastocyst
216 formation rate, the apoptotic nucleus indices and total cell number without electroporation
217 treatment are shown in Table 1. Supplementation of CGA at 50 μM/L during IVM culture
218 significantly increased the maturation rate of oocytes and the total cell number in
219 blastocysts compared to those of the oocytes without CGA treatment ($P < 0.05$). Porcine
220 blastocysts derived from oocytes treated with 50 μM/L CGA showed significantly lower
221 apoptotic nucleus indices than those of blastocysts without CGA treatment ($P < 0.05$).
222 However, an increase of CGA to 100 μM/L did not improve the developmental
223 competence of oocytes. There were no differences in blastocyst formation rate between
224 the four groups.

225

226 **In vitro development and quality of electroporated porcine embryos derived from**
227 **oocytes matured with various concentrations of CGA**

228 The effects of CGA supplementation during IVM culture on the development and
229 quality of embryos electroporated after *in vitro* fertilization are shown in Table 2. The
230 blastocyst formation rate in the 50 μM/L CGA treatment group was significantly higher
231 than that in the CGA-untreated and the 10 μM/L CGA-treated group ($P < 0.05$).
232 Supplementation of CGA at 50 μM/L during IVM culture also significantly decreased the
233 apoptotic nucleus index of blastocysts compared to that of the untreated group ($P < 0.05$).
234 No differences in total cell number in blastocysts were found among the groups.

235

236 DISCUSSION

237

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

249 *In vitro* environments for embryo culture systems contain a higher oxygen
250 concentration than those of *in vivo* environments within the lumen of the female
251 reproductive tract (Mastroianni and Jones 1965). High oxygen concentration is
252 predominantly associated with an increase in the production of ROS and leads to
253 oxidative stress in the oocytes and embryos (Agarwal et al. 2006, Agarwal et al. 2003).
254 The balance between oxygen factors and antioxidants in the culture medium is therefore
255 very important to drive the success of *in vitro* production (IVP) system. Administration
256 of CGA, a dietary polyphenol shown to have antioxidant activity (Rice-Evans *et al.* 1996;
257 Sato *et al.* 2011), to the maturation medium has recently shown the potential to improve
258 porcine IVP (Nguyen *et al.* 2017). To confirm whether the CGA has any adverse effect
259 on the developmental competence of the embryo, we examined the quality and
260 development of porcine embryos without electroporation by CGA supplementation with

different concentrations during *in vitro* maturation culture. Our results showed that there were no adverse effects of CGA supplementation at a concentration of 50 μM/L 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 (Shil *et al.* 2005; Kuznetsov *et al.* 2011) 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/L 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/L CGA is advantageous to porcine IVP system. When porcine zygotes from oocytes matured with or without 50 μM/L CGA were electroporated with Cas9 mRNA and sgRNA targeting site 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 (You & Chan 2015; Nguyen *et al.* 2017). An increase in the number of apoptotic embryos with DNA fragmentation appears to be related to increasing H_2O_2 concentrations (Yang *et al.* 1998; Lee & Yeung 2006). In the present study, the improvement of porcine embryo development and quality by CGA supplementation during IVM culture might be

287 explained through its antioxidant activity. The exposure to CGA during *in vitro* maturation
288 culture may potentially decrease H_2O_2-induced apoptotic cell death by up-regulating anti-
289 apoptotic proteins and preventing H_2O_2-induced caspase activation (Kim *et al.* 2012;
290 Rebai *et al.* 2017), leading to the depletion of ROS in the electroporated porcine embryos.
291 The oocytes and embryos are then protected from oxidative stress that could be induced
292 by either a high oxygen concentration under *in vitro* culture conditions or electroporation
293 treatment.

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

299

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301

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308

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