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Title:

Chlorogenic acid supplementation during *in vitro* maturation improves maturation, fertilisation, and developmental competence of porcine oocytes

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Running title:

Effects of chlorogenic acid on porcine oocytes

Abstract

Chlorogenic acid (CGA) is a quinic acid conjugate of caffeic acid, and a phytochemical found in many fruits and beverages that acts as an antioxidant. The present study investigated the effects of CGA supplementation during *in vitro* maturation, 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 fertilised, and cultured *in vitro* for 7 d. The rates of maturation, fertilisation, and blastocyst formation of oocytes matured with 50 μM CGA was significantly ($p < 0.05$) higher than those of the control oocytes. Hydrogen peroxide (H_2O_2) is one of the reactive oxygen species and induces DNA damage in porcine oocytes. When oocytes were matured 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, fertilisation, 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 *in vitro* maturation with 50 μM CGA is particularly beneficial to *in vitro* production of porcine embryos and protects oocytes from DNA damage induced by oxidative stress. Supplementation of CGA to the maturation medium has a potential to improve porcine IVP system.

Keywords: antioxidative stress, embryo, DNA fragmentation, chlorogenic acid, developmental competence

1. Introduction

The *in vitro* production (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 a suitable source of cells and organs for xenotransplantation (Critser et al. 2009; Ramsoondar et al. 2009; Samiec and Skrzyszowska 2011a), and a transgenic animal capable of producing specific proteins that it shares with humans (Takahagi et al. 2005; Pan et al. 2010; Samiec and Skrzyszowska 2011b).

Currently used IVP systems comprise three major steps: in vitro maturation (IVM), *in vitro* fertilisation (IVF) or somatic cell nuclear transfer (SCNT), and *in vitro* culture (IVC) of fertilised or cloned embryos (Somfai and Hirao 2011; Samiec and Skrzyszowska 2012a, 2013; Samiec et al. 2015). Although many attempts have been made to produce high-quality IVF- or SCNT-derived embryos, their developmental competence in the pig remains insufficient and lower than that of in vivo-derived embryos of the pig and of other mammalian species, such as cattle or mice (Kikuchi et al. 1999, 2002; Dang-Nguyen et al. 2011; 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 (Yoshioka et al. 2002; Pang et al. 2013; Samiec and Skrzyszowska 2012b, 2014). It has been established that the oxygen concentration within the lumen of the female reproductive tract (*in vivo*) is about 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 reactive oxygen species (ROS) and in turn, increased oxidative

74 stress on the oocytes (Agarwal et al. 2003; Agarwal et al. 2006). Therefore, antioxidants
75 might be beneficial additives that could protect *in vitro* oocytes from stress and thereby
76 improve developmental competence of oocytes.
77 Chlorogenic acid (CGA) is a quinic acid conjugate of caffeic acid (Gonthier et al.
78 2006) found at high levels in coffee beans and various sources of fruit including
79 strawberries, blueberries, eggplants, and tomatoes (Mahmood et al. 2012; Cho et al. 2010).
80 As a phytochemical, several health benefits of CGA have been demonstrated, including
81 antioxidative (Hoelzl et al. 2010), hepatoprotective (Xu et al. 2010), anti-obesity (Cho et
82 al. 2010), anti-inflammatory, and antinociceptive effects (Kupeli Akkol et al. 2012).-CGA
83 appears to have similar antioxidant potential to caffeic acid, as assessed by the oxygen
84 radical absorbance capacity (ORAC) (Ishimoto et al. 2012). In the present study, we
85 investigated the antioxidant effects of CGA supplementation during IVM on the meiotic
86 and developmental competence of porcine oocytes.
87
88 **2. Materials and Methods**
89 There were no live animals used in this study, so no ethical approval was required.
90
91 *2.1. In vitro maturation and assessment*
92 Porcine ovaries were obtained from approximately 6-months old gilts at a local
93 slaughterhouse and were transported within 3 h to the laboratory in physiological saline
94 at 30°C. Ovaries were washed three times with modified phosphate-buffered saline (m-
95 PBS; Nihonzenyaku, Fukushima, Japan) supplemented with 100 IU/ml penicillin G
96 potassium (Meiji, Tokyo, Japan) and 0.1 mg/ml streptomycin sulfate (Meiji). The
97 cumulus-oocyte complexes (COCs) were collected from ovaries. The follicles of ovarian
98 surface were sliced using a surgical blade on the sterilised dish. Only COCs with a

99 uniformly dark-pigmented ooplasm and intact cumulus cell masses were collected under
100 a stereomicroscope. Approximately 50 COCs were then cultured in 500 µl of maturation
101 medium, consisting of 25 mM HEPES tissue culture medium 199 with Earle's salts (TCM
102 199; #12340, Invitrogen Co., Carlsbad, CA, USA), supplemented with 10% (v/v) porcine
103 follicular fluid; 0.6 mM cysteine (Sigma-Aldrich, St. Louis, MO, USA); 50 µM sodium
104 pyruvate (Sigma-Aldrich); 2 mg/ml D-sorbitol (Wako Pure Chemical Industries Ltd.); 1
105 µg/ml 17β-estradiol (Sigma-Aldrich); 10 IU/ml equine chorionic gonadotropin (Kyoritu
106 Seiyaku, Tokyo, Japan); 10 IU/ml human chorionic gonadotropin (Kyoritu Seiyaku); and
107 50 µg/ml gentamicin (Sigma-Aldrich), for 22 h in 4-well dishes (Nunc A/S, Roskilde,
108 Denmark). Subsequently, the COCs were transferred into maturation medium without
109 hormone supplementation and cultured for an additional 22 h. The incubation of COCs
110 was conducted at 39°C in a humidified incubator containing 5% CO_2 in air.
111 To assess the meiotic status of oocytes following IVM, some oocytes were denuded,
112 fixed, and permeabilised in Dulbecco's PBS (DPBS; Invitrogen) supplemented with 3.7%
113 (w/v) paraformaldehyde and 1% (v/v) Triton X-100 (Sigma-Aldrich) at 25°C for 15 min.
114 Permeabilised oocytes were then placed on glass slides and stained with 1.9 mM
115 bisbenzimide (Hoechst 33342; Sigma-Aldrich), before being covered with coverslips.
116 After overnight incubation at 4°C, the oocytes were examined by fluorescence
117 microscopy. Based on their chromatin configuration, they were classified as ‘germinal
118 vesicle,’ ‘condensed chromatin,’ ‘metaphase I,’ or ‘metaphase II’ (Wongsrikeao et al.
119 2004). Oocytes with the diffusely stained cytoplasmic characteristics of nonviable cells,
120 and those in which chromatin was unidentifiable or not visible were classified as
121 ‘degenerated.’
122
123 *2.2. IVF and assessment of fertilisation*

IVF was performed according to methods described by Namula et al. (2013) with minor modifications. Frozen-thawed spermatozoa were transferred into 5 ml of fertilisation 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 × *g* for 5 min. The pellets of spermatozoa were resuspended in fertilisation medium to obtain a final sperm concentration of 1×10^7 cells/ml. Some of the spermatozoa (50 µl) were added to 50 µl of fertilisation medium containing 10–20 matured oocytes. The final sperm concentration was adjusted to 5×10^6 cells/ml. The oocytes were co-incubated with spermatozoa for 12 h at 39°C in a humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2. 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 fertilisation 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 phase contrast microscopy. Oocytes containing both female and male pronuclei were considered fertilised, 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).

2.3. 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.) 72 h after insemination, and cultured for an additional 5 days to

149 evaluate their ability to develop to the blastocyst stage. To evaluate the development stage
150 of fertilised zygotes, all embryos were fixed on day 7 (day 0; insemination) and were
151 stained with Hoechst 33342 to assess the quality of embryos by counting cell number.
152
153 *2.4. Experiment 1*
154 To evaluate the effects of CGA supplementation during IVM culture on the *in vitro*
155 maturation, fertilisation, and development of porcine oocytes, the COCs were cultured in
156 maturation medium supplemented with 10, 50, 100, and 200 μM CGA (Sigma-Aldrich).
157 As a control, COCs were cultured in maturation medium without CGA. After maturation
158 culture for 44 h, the COCs were fertilised *in vitro* and cultured continuously *in vitro* as
159 described above.
160
161 *2.5. Experiment 2*
162 To assess the protective effect of CGA on hydrogen peroxide (H_2O_2)-induced DNA
163 damage in porcine oocytes, the COCs were exposed to 1 mM H_2O_2 (Do et al. 2015) in
164 maturation medium supplemented either with or without CGA (50 μM) during IVM.
165 After maturation at 39°C for 44 h, the oocytes were denuded, fixed and then evaluated
166 for nuclear status and DNA fragmentation, using a combined technique for simultaneous
167 nuclear staining and terminal deoxynucleotidyl transferase dUTP nick end labeling
168 (TUNEL), modified from procedures previously described by Otoi et al. (1999). Oocytes
169 were fixed overnight at 4°C in 3.7% (w/v) paraformaldehyde diluted in PBS. After
170 fixation, the oocytes were permeabilized in PBS containing 0.1% (v/v) Triton X-100 for
171 40 min. They were subsequently incubated overnight at 4°C in PBS containing 10 mg/ml
172 bovine serum albumin (blocking solution). They were then incubated in fluorescein-
173 conjugated 2-deoxyuridine 5-triphosphate and TUNEL reagent (Roche Diagnostics Corp.,

174 Tokyo, Japan) for 1 h at 38.5°C. After TUNEL staining, the oocytes were counterstained
175 with 1 μg/ml 4′6-diamidino-2-phenylindole (DAPI) (Invitrogen) for 10 min to assess the
176 meiotic status of oocytes. They were then treated with an anti-bleaching solution (Slow-
177 Fade; Molecular Probes Inc., Eugene, OR, USA), mounted on glass slides, and then sealed
178 with clear nail polish. Labelled oocytes were examined using an epifluorescence
179 microscope (Eclipse 80i, Nikon). Apoptotic nuclei showed condensed and fragmented
180 morphology (Brison and Schultz 1997; Pawlak et al. 2011). The apoptotic rate was
181 calculated by dividing the number of oocytes containing DNA-fragmented nuclei
182 (labelled by TUNEL) by the total number of oocytes.
183
184 *2.6. Experiment 3*
185 To compare the supplementation effects of CGA and caffeic acid during IVM culture
186 on the *in vitro* maturation, fertilisation, and development of porcine oocytes, the COCs
187 were matured in the medium supplemented with 10, 50 and 100 μM caffeic acid (Sigma-
188 Aldrich). As a control, COCs were cultured in maturation medium supplemented with 50
189 μM CGA. The concentration of CGA (50 μM) found most suitable for the development
190 of embryos in Experiment 1 was used in this experiment.
191
192 *2.7. Statistical analysis*
193 Experiments were repeated five times for oocytes matured with CGA, and four times
194 for oocytes exposed to H_2O_2 and oocytes matured with caffeic acid. Percentages of
195 matured oocytes, fertilised oocytes, monospermy, cleaved embryos, embryos develop to
196 the blastocyst stage, and apoptotic oocytes were subjected to arcsine transformation
197 before analysis of variance (ANOVA). The transformed data were tested by ANOVA,
198 followed by Fisher's protected least significant difference (LSD) test, using the StatView

199 software (Abacus Concepts, Berkeley, CA, USA). Differences with a probability value
200 (p) of 0.05 or less were considered to be statistically significant.
201
202 **3. Results**
203
204 *3.1. Effects of CGA supplementation during IVM on maturation, fertilisation, and*
205 *development of porcine oocytes*
206 As shown in Table 1, the maturation rate of oocytes matured with 50 μM CGA (78.8
207 ± 3.8%) was significantly increased, compared to the control (63.1 ± 3.0%) and two other
208 CGA concentrations (64.6 ± 7.2% and 65.2 ± 4.7% for 10 and 200 μM, respectively) (p
209 < 0.05). The fertilisation rate (60.9 ± 4.3%) of oocytes matured with 50 μM CGA during
210 IVM was significantly higher (p < 0.05) than that of oocytes matured without CGA (39.1
211 ± 1.9%). Moreover, the blastocyst formation rate of oocytes matured with 50 μM CGA
212 (21.6 ± 2.2%) was significantly higher (p < 0.05) than that of the control (9.3 ± 1.9%) and
213 all other concentrations of CGA (13.9 ± 3.1%, 14.4 ± 2.9%, and 12.2 ± 1.6% for 10, 100,
214 and 200 μM, respectively). However, no effects of CGA treatment were observed on the
215 monospermy and cleavage rates of embryos.
216
217 *3.2. Effects of CGA supplementation during IVM on maturation rate and DNA*
218 *fragmentation of porcine oocytes exposed to H_2O_2*
219 As shown in Fig. 1, exposure of oocytes to 1 mM H_2O_2 during IVM significantly
220 reduced the maturation rate (1.4 ± 0.8%) compared to that of non-exposed oocytes (68.6
221 ± 2.9%) (p < 0.01). Supplementation of the maturation medium with 50 μM CGA
222 significantly improved the maturation rate of oocytes exposed to H_2O_2 (46.2 ± 4.5%)
223 (p < 0.01). When oocytes that had been matured without CGA were exposed to 1 mM

224 H_2O_2 during IVM, the proportion of DNA-fragmented nuclei (62.9 ± 6.8%) was
225 significantly higher ($p < 0.01$) than that of the non-exposed group (3.9 ± 1.8%).
226 However, CGA treatment significantly reduced the proportion of DNA-fragmented nuclei
227 (30.2 ± 5.9%) ($p < 0.05$).
228
229 *3.3. Effects of caffeic acid supplementation during IVM on maturation, fertilisation, and*
230 *development of porcine oocytes*
231 As shown in Table 2, the rates of maturation, fertilisation, monospermy, and
232 blastocyst formation of oocytes matured with 10 and 50 μM caffeic acid were similar to
233 the control oocytes matured with 50 μM CGA. In contrast, the rates of maturation,
234 fertilisation, monospermy, and blastocyst formation of oocytes matured with 100 μM
235 caffeic acid were significantly lower ($p < 0.05$) than those of the control oocytes. There
236 were no differences in the cleavage rates of oocytes after IVF among the groups.
237
238 **4. Discussion**
239 In the present study, we confirmed the antioxidant potential of CGA. Firstly, we
240 found that supplementation of the maturation medium with 50 μM CGA significantly
241 improved the rates of maturation, fertilisation, and blastocyst formation of oocytes.
242 Furthermore, the supplementation effect of caffeic acid during IVM was similar to the
243 CGA which is a quinic acid conjugate of caffeic acid.
244 Oxidative stress poses a threat to oocytes and embryos *in vitro*, when these cells are
245 removed from their natural habitat into one that lacks maternal antioxidant factors. The
246 oxygen concentration in a standard IVP system is guessed to be higher than that in the
247 female reproductive tract (Mastroianni and Jones 1965). Moreover, high oxygen
248 concentrations associated with *in vitro* conditions results in increased oxidative stress that

249 has been reported to have negative effects on the quality of embryos, and might lead to
250 an early block and retardation of embryonic development (Agarwal et al. 2003; Agarwal
251 et al. 2006). Considerable evidence in animal studies indicates that supplementation of
252 culture media with antioxidants, vitamins C and E, amino acids, or ROS scavengers can
253 be alternative treatment strategies that help to reduce oxidative stress and can be
254 beneficial to embryonic survival and blastocyst formation rates (Taylor 2001). To date, a
255 variety of media have been developed for the porcine IVP system. Each media system
256 requires a balance between oxygen factors and antioxidants. Therefore, it is also
257 necessary to investigate the optimal concentration of the supplemented antioxidant. Our
258 results indicate that 50 μM is the optimal concentration of CGA supplementation during
259 porcine IVM. To our knowledge, the present study was the first to employ this application
260 of CGA to *in vitro* development of the porcine oocyte. CGA is a quinic acid ester of
261 caffeic acid which has the antioxidant ability with respect to their capability to quench a
262 reactive oxygen species (Foley et al. 1999). Rice-Evans et al. (1996) demonstrated that
263 there are no differences between caffeic acid and CGA in their inhibitory effects on LDL
264 oxidation. In this study, we demonstrated that the effects of 50 μM CGA supplementation
265 to the maturation medium were comparable to 10 and 50 μM caffeic acid supplementation
266 in porcine oocytes. It has demonstrated that 50 μM of caffeic acid protected human and
267 mice cells against oxidative stress *in vitro* (Nardini et al. 1998; Lapidot et al. 2002). These
268 studies support our results and the antioxidant ability of CGA.
269 The members of ROS family include H_2O_2 that is more stable than O_2 and can be
270 readily diffused through cell membranes. A direct relationship between increased
271 concentrations of H_2O_2 and apoptosis has been observed in fragmented embryos and
272 blastocysts (Lee and Yeung 2006; Yang et al. 1998). Pierce et al. (1991) have also
273 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 defenses (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 of porcine IVP system.

In conclusion, CGA is an effective antioxidant that improves the maturation, fertilisation, 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 *in vitro* production of porcine embryos as an antioxidant supplemented to the porcine maturation medium. It appears that CGA supplementation

298 in the maturation medium improves the porcine IVP system, which can be beneficial to
299 further developments in biotechnology.
300
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306
307 **6. Conflict of interest statement**
308 The authors declare no conflicts of interest.
309
310 **7. Author contributions**
311 T.-V.N., F.T. and T.O. conceived the study and wrote the manuscript. T.-V.N. performed
312 most of the experiment and wrote the most part of the manuscript. T.O. designed the study,
313 coordinated all of the experiments and reviewed the manuscript. F.T. participated in the
314 laboratorial work, revised the manuscript and contributed to the statistical analysis.
315 L.T.K.D and participated in the laboratorial work. Y. S., M.T. and T.V.N. supported the
316 experimental conception and reviewed the manuscript. All authors read and accepted the
317 manuscript.

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462 **9. Figure legend**

463 **Figure 1**. Effects of chlorogenic acid (CGA; 50 μM) supplementation during *in vitro*
464 maturation on the maturation rate (A) and the proportion of DNA-fragmented nuclei (B)
465 of porcine oocytes exposed to 1.0 mM H_2O_2. Oocytes matured without CGA served as
466 the control group. Each bar presents the mean value ± SEM (n = 4 replications, each with
467 100–110 oocytes per treatment). Bars with different letters differ significantly ($p < 0.05$).

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Table 1. Effects of chlorogenic acid (CGA) supplementation during *in vitro* maturation culture on the maturation, fertilisation and development of porcine oocytes*

Concentration of CGA (μM)	Number of examined oocytes	Number (%)** of matured oocytes	Number of examined oocytes	Number of oocytes		Number of examined embryos	Number of embryos	
				Fertilised (%)	Monospermy (%)***		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)[ab]	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]

* Five replicated trials were carried out.

** Percentages are expressed as mean ± SEM.

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

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

Table 2. Effects of caffeic acid supplementation during *in vitro* maturation culture on the maturation, fertilisation and development of porcine oocytes*

Concentration of caffeic acid (μM)	Number of examined oocytes	Number (%)*** of matured oocytes	Number of examined oocytes	Number of oocytes		Number of examined embryos	Number of embryos	
				Fertilised (%)	Monospermy (%)****		Cleaved (%)	Developed to blastocysts (%)
0**	54	40 (73.9 ± 2.7)[a]	60	44 (73.3 ± 4.7)[ab]	37 (61.7 ± 1.7)[ab]	145	130 (89.7 ± 1.5)	14 (9.7 ± 1.8)[a]
10	52	37 (71.2 ± 1.9)[ab]	59	42 (71.2 ± 1.6)[ab]	37 (62.7 ± 1.7)[a]	150	133 (88.7 ± 2.1)	11 (7.3 ± 2.1)[ab]
50	52	40 (76.9 ± 3.1)[a]	58	44 (75.9 ± 4.4)[a]	38 (65.5 ± 2.8)[a]	139	124 (89.2 ± 1.1)	6 (4.3 ± 2.6)[ab]
100	55	35 (63.8 ± 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]

* Four replicated trials were carried out.

** As a control, porcine oocytes were matured with 50 μM chlorogenic acid.

*** Percentages are expressed as mean ± SEM.

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

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