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8 Title:

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

11

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19 *Running title:*

20 Effects of chlorogenic acid on porcine oocytes

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24

25 **Abstract**

26 Chlorogenic acid (CGA) is a quinic acid conjugate of caffeic acid, and a  
27 phytochemical found in many fruits and beverages that acts as an antioxidant. The present  
28 study investigated the effects of CGA supplementation during *in vitro* maturation, on *in*  
29 *vitro* development of porcine oocytes, in order to improve the porcine *in vitro* production  
30 (IVP) system. Oocytes were matured either without (control) or with CGA (10, 50, 100,  
31 and 200  $\mu\text{M}$ ). Subsequently, the matured oocytes were fertilised, and cultured *in vitro* for  
32 7 d. The rates of maturation, fertilisation, and blastocyst formation of oocytes matured  
33 with 50  $\mu\text{M}$  CGA was significantly ( $p < 0.05$ ) higher than those of the control oocytes.  
34 Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) is one of the reactive oxygen species and induces DNA  
35 damage in porcine oocytes. When oocytes were matured with 1 mM  $\text{H}_2\text{O}_2$  to assess the  
36 protective effect of CGA, 50  $\mu\text{M}$  CGA supplementation improved the maturation rate and  
37 the proportion of DNA-fragmented nuclei in oocytes compared with control oocytes  
38 matured without CGA. Moreover, when oocytes were matured with either 50  $\mu\text{M}$  CGA  
39 (control) or caffeic acid (10, 50, and 100  $\mu\text{M}$ ), the rates of maturation, fertilisation, and  
40 the blastocyst formation of oocytes matured with 50  $\mu\text{M}$  CGA were similar to those of  
41 oocytes matured with 10 and 50  $\mu\text{M}$  caffeic acid. Our results suggest that CGA has  
42 comparable effects to caffeic acid, and *in vitro* maturation with 50  $\mu\text{M}$  CGA is  
43 particularly beneficial to *in vitro* production of porcine embryos and protects oocytes  
44 from DNA damage induced by oxidative stress. Supplementation of CGA to the  
45 maturation medium has a potential to improve porcine IVP system.

46

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

49 **1. Introduction**

50 The *in vitro* production (IVP) of porcine embryos is of interest to the scientific  
51 community because of its capacity to produce large quantities of matured oocytes and  
52 embryos that are crucial to basic science (such as reproductive physiology), as well as  
53 advances in biotechnology and biomedical research. Furthermore, with respect to  
54 anatomy and physiology, the pig is similar to humans, and is therefore considered a  
55 suitable source of cells and organs for xenotransplantation (Critser et al. 2009;  
56 Ramsoondar et al. 2009; Samiec and Skrzyszowska 2011a), and a transgenic animal  
57 capable of producing specific proteins that it shares with humans (Takahagi et al. 2005;  
58 Pan et al. 2010; Samiec and Skrzyszowska 2011b).

59 Currently used IVP systems comprise three major steps: *in vitro* maturation (IVM),  
60 *in vitro* fertilisation (IVF) or somatic cell nuclear transfer (SCNT), and *in vitro* culture  
61 (IVC) of fertilised or cloned embryos (Somfai and Hirao 2011; Samiec and Skrzyszowska  
62 2012a, 2013; Samiec et al. 2015). Although many attempts have been made to produce  
63 high-quality IVF- or SCNT-derived embryos, their developmental competence in the pig  
64 remains insufficient and lower than that of *in vivo*-derived embryos of the pig and of  
65 other mammalian species, such as cattle or mice (Kikuchi et al. 1999, 2002; Dang-Nguyen  
66 et al. 2011; Samiec et al. 2012). Thus, it is crucial to improve the developmental  
67 competence of IVF- or SCNT-derived embryos because this inefficiency diminishes its  
68 application to further studies that require high-quality embryos (Yoshioka et al. 2002;  
69 Pang et al. 2013; Samiec and Skrzyszowska 2012b, 2014). It has been established that the  
70 oxygen concentration within the lumen of the female reproductive tract (*in vivo*) is about  
71 one third that found under standard *in vitro* conditions (Mastroianni and Jones 1965).  
72 Generally, the high oxygen concentration associated with *in vitro* conditions results in  
73 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  $\mu$ l 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  $\mu$ M sodium  
104 pyruvate (Sigma-Aldrich); 2 mg/ml D-sorbitol (Wako Pure Chemical Industries Ltd.); 1  
105  $\mu$ g/ml 17 $\beta$ -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  $\mu$ g/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<sub>2</sub> 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 bisbenzimidazole (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*

124 IVF was performed according to methods described by Namula et al. (2013) with  
125 minor modifications. Frozen-thawed spermatozoa were transferred into 5 ml of  
126 fertilisation medium (PFM; Research Institute for the Functional Peptides Co., Yamagata,  
127 Japan) in a 15 ml test tube, and were then washed by centrifugation at  $500 \times g$  for 5 min.  
128 The pellets of spermatozoa were resuspended in fertilisation medium to obtain a final  
129 sperm concentration of  $1 \times 10^7$  cells/ml. Some of the spermatozoa (50  $\mu$ l) were added to  
130 50  $\mu$ l of fertilisation medium containing 10–20 matured oocytes. The final sperm  
131 concentration was adjusted to  $5 \times 10^6$  cells/ml. The oocytes were co-incubated with  
132 spermatozoa for 12 h at 39°C in a humidified incubator containing 5% CO<sub>2</sub>, 5% O<sub>2</sub>, and  
133 90% N<sub>2</sub>. Following co-incubation with spermatozoa for 12 h, the presumed zygotes were  
134 denuded from the cumulus cells and attached spermatozoa by mechanical pipetting.

135 To assess fertilisation of the oocytes, some denuded zygotes were mounted on glass  
136 slides and fixed with acetic acid:ethanol (1:3 v/v) for 48–72 h. The fixed zygotes were  
137 stained with acetic orcein (1% orcein in 45% acetic acid) and examined by phase contrast  
138 microscopy. Oocytes containing both female and male pronuclei were considered  
139 fertilised, and were categorized as normal or polyspermic, based on the number of swollen  
140 sperm heads and/or pronuclei in the cytoplasm (Do et al. 2015).

141

### 142 2.3. *IVC and assessment of blastocyst quality*

143 The remaining denuded zygotes were subsequently transferred to 100  $\mu$ l droplets of  
144 PZM-5 (Research Institute for the Functional Peptides Co.). Each droplet contained  
145 approximately 10 presumed zygotes. The zygotes were cultured continuously *in vitro* at  
146 39°C in a humidified incubator containing 5% CO<sub>2</sub>, 5% O<sub>2</sub>, and 90% N<sub>2</sub>. All of the  
147 cleaved embryos were transferred into 100  $\mu$ l droplets of PBM (Research Institute for the  
148 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  $\mu$ M 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  $\mu$ M) 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<sub>2</sub>O<sub>2</sub> 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  $\mu$ M CGA (78.8  
207  $\pm$  3.8%) was significantly increased, compared to the control (63.1  $\pm$  3.0%) and two other  
208 CGA concentrations (64.6  $\pm$  7.2% and 65.2  $\pm$  4.7% for 10 and 200  $\mu$ M, respectively) ( $p$   
209  $<$  0.05). The fertilisation rate (60.9  $\pm$  4.3%) of oocytes matured with 50  $\mu$ M CGA during  
210 IVM was significantly higher ( $p <$  0.05) than that of oocytes matured without CGA (39.1  
211  $\pm$  1.9%). Moreover, the blastocyst formation rate of oocytes matured with 50  $\mu$ M CGA  
212 (21.6  $\pm$  2.2%) was significantly higher ( $p <$  0.05) than that of the control (9.3  $\pm$  1.9%) and  
213 all other concentrations of CGA (13.9  $\pm$  3.1%, 14.4  $\pm$  2.9%, and 12.2  $\pm$  1.6% for 10, 100,  
214 and 200  $\mu$ M, 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<sub>2</sub>O<sub>2</sub>*

219 As shown in Fig. 1, exposure of oocytes to 1 mM H<sub>2</sub>O<sub>2</sub> during IVM significantly  
220 reduced the maturation rate (1.4  $\pm$  0.8%) compared to that of non-exposed oocytes (68.6  
221  $\pm$  2.9%) ( $p <$  0.01). Supplementation of the maturation medium with 50  $\mu$ M CGA  
222 significantly improved the maturation rate of oocytes exposed to H<sub>2</sub>O<sub>2</sub> (46.2  $\pm$  4.5%)  
223 ( $p <$  0.01). When oocytes that had been matured without CGA were exposed to 1 mM

224 H<sub>2</sub>O<sub>2</sub> during IVM, the proportion of DNA-fragmented nuclei ( $62.9 \pm 6.8\%$ ) was  
225 significantly higher ( $p < 0.01$ ) than that of the non-exposed group ( $3.9 \pm 1.8\%$ ).  
226 However, CGA treatment significantly reduced the proportion of DNA-fragmented nuclei  
227 ( $30.2 \pm 5.9\%$ ) ( $p < 0.05$ ).

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  $\mu\text{M}$  caffeic acid were similar to  
233 the control oocytes matured with 50  $\mu\text{M}$  CGA. In contrast, the rates of maturation,  
234 fertilisation, monospermy, and blastocyst formation of oocytes matured with 100  $\mu\text{M}$   
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  $\mu\text{M}$  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  $\mu\text{M}$  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  $\mu\text{M}$  CGA supplementation  
265 to the maturation medium were comparable to 10 and 50  $\mu\text{M}$  caffeic acid supplementation  
266 in porcine oocytes. It has demonstrated that 50  $\mu\text{M}$  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  $\text{H}_2\text{O}_2$  that is more stable than  $\text{O}_2$  and can be  
270 readily diffused through cell membranes. A direct relationship between increased  
271 concentrations of  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  as a mediator of apoptosis in the blastocyst. Moreover, the maturation of

274 oocytes and development of embryos are also affected by increased levels of ROS or  
275 reduced antioxidant defenses (Blondin et al. 1997; Harvey et al. 2002). Therefore,  
276 apoptosis levels of oocytes and embryos can be useful indicators of oocyte quality and  
277 embryonic development (Brison and Schultz 1997; Tatemoto et al. 2000). In the present  
278 study, we confirmed the deleterious effects of H<sub>2</sub>O<sub>2</sub> on the induction of DNA  
279 fragmentation in porcine oocytes matured *in vitro* that had been demonstrated in our  
280 previous study (Do et al. 2015). We also found that when oocytes were exposed to H<sub>2</sub>O<sub>2</sub>  
281 during IVM, CGA effectively restored the oocyte maturation rate and protected oocytes  
282 from DNA fragmentation. These results confirmed the antioxidant effect of CGA on  
283 porcine oocytes exposed to H<sub>2</sub>O<sub>2</sub> during IVM; therefore, CGA has a positive effect by  
284 preventing apoptosis and improving the quality of oocytes matured *in vitro*. Other  
285 antioxidants and H<sub>2</sub>O<sub>2</sub> scavengers also have a positive effect on preventing H<sub>2</sub>O<sub>2</sub> damage  
286 during porcine IVP. Melatonin is one of the antioxidants and free radical scavengers  
287 which has beneficial effects on nuclear and cytoplasmic maturation during porcine IVM  
288 (Kang et al. 2009). Yazaki et al. (2013) have reported L-carnitine, an H<sub>2</sub>O<sub>2</sub> scavenger,  
289 improves H<sub>2</sub>O<sub>2</sub>-induced impairment of nuclear maturation in porcine oocytes. Vitamin-E  
290 is also one of the antioxidants. Vitamin-E suppressed oxidative damage and improved  
291 their developmental ability of porcine oocytes (Kitagawa et al. 2004). These studies  
292 support the antioxidant effect of CGA on the improvement of porcine IVP system.

293 In conclusion, CGA is an effective antioxidant that improves the maturation,  
294 fertilisation, and developmental competence of porcine IVP oocytes, and protects oocytes  
295 from DNA fragmentation caused by H<sub>2</sub>O<sub>2</sub> exposure. CGA also has comparable effects to  
296 caffeic acid on improving *in vitro* production of porcine embryos as an antioxidant  
297 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  $\mu$ M) 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<sub>2</sub>O<sub>2</sub>. Oocytes matured without CGA served as  
466 the control group. Each bar presents the mean value  $\pm$  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 ( $\mu\text{M}$ ) | 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 $\pm$ 3.0) <sup>a</sup>   | 92                         | 37 (39.1 $\pm$ 1.9) <sup>a</sup>  | 25 (74.5 $\pm$ 7.8) | 209                        | 157 (75.7 $\pm$ 4.7) | 19 (9.3 $\pm$ 1.9) <sup>a</sup>  |
| 10                                     | 133                        | 93 (64.6 $\pm$ 7.2) <sup>a</sup>   | 112                        | 58 (54.0 $\pm$ 4.0) <sup>ab</sup> | 36 (60.7 $\pm$ 5.5) | 223                        | 179 (80.6 $\pm$ 4.3) | 30 (13.9 $\pm$ 3.1) <sup>a</sup> |
| 50                                     | 151                        | 118 (78.8 $\pm$ 3.8) <sup>b</sup>  | 108                        | 63 (60.9 $\pm$ 4.3) <sup>b</sup>  | 42 (78.9 $\pm$ 2.9) | 211                        | 182 (86.7 $\pm$ 1.3) | 45 (21.6 $\pm$ 2.2) <sup>b</sup> |
| 100                                    | 147                        | 112 (75.9 $\pm$ 2.5) <sup>ab</sup> | 95                         | 45 (49.8 $\pm$ 7.9) <sup>ab</sup> | 24 (64.8 $\pm$ 6.2) | 216                        | 182 (84.5 $\pm$ 3.4) | 30 (14.4 $\pm$ 2.9) <sup>a</sup> |
| 200                                    | 151                        | 104 (65.2 $\pm$ 4.7) <sup>a</sup>  | 97                         | 44 (47.2 $\pm$ 4.8) <sup>ab</sup> | 23 (64.0 $\pm$ 6.0) | 223                        | 175 (78.7 $\pm$ 1.4) | 27 (12.2 $\pm$ 1.6) <sup>a</sup> |

\* Five replicated trials were carried out.

\*\* Percentages are expressed as mean  $\pm$  SEM.

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

<sup>a, b</sup>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 ( $\mu\text{M}$ ) | 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 $\pm$ 2.7) <sup>a</sup>  | 60                         | 44 (73.3 $\pm$ 4.7) <sup>ab</sup> | 37 (61.7 $\pm$ 1.7) <sup>ab</sup> | 145                        | 130 (89.7 $\pm$ 1.5) | 14 (9.7 $\pm$ 1.8) <sup>a</sup>  |
| 10  | 52                         | 37 (71.2 $\pm$ 1.9) <sup>ab</sup> | 59                         | 42 (71.2 $\pm$ 1.6) <sup>ab</sup> | 37 (62.7 $\pm$ 1.7) <sup>a</sup>  | 150                        | 133 (88.7 $\pm$ 2.1) | 11 (7.3 $\pm$ 2.1) <sup>ab</sup> |
| 50  | 52                         | 40 (76.9 $\pm$ 3.1) <sup>a</sup>  | 58                         | 44 (75.9 $\pm$ 4.4) <sup>a</sup>  | 38 (65.5 $\pm$ 2.8) <sup>a</sup>  | 139                        | 124 (89.2 $\pm$ 1.1) | 6 (4.3 $\pm$ 2.6) <sup>ab</sup>  |
| 100   | 55                         | 35 (63.8 $\pm$ 2.0) <sup>b</sup>  | 57                         | 35 (61.4 $\pm$ 3.4) <sup>b</sup>  | 31 (54.7 $\pm$ 3.2) <sup>b</sup>  | 143                        | 121 (84.6 $\pm$ 2.0) | 4 (2.8 $\pm$ 1.5) <sup>b</sup>   |

\* Four replicated trials were carried out.

\*\* As a control, porcine oocytes were matured with 50  $\mu\text{M}$  chlorogenic acid.

\*\*\* Percentages are expressed as mean  $\pm$  SEM.

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

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