

1 **Title:**

2 **Effects of chlorogenic acid and caffeic acid on the quality of frozen-thawed boar**
3 **sperm**

4
5
6 Running title:

7 Chlorogenic and caffeic acid supplementation during sperm freezing

8
9 Authors:

10 Zhao Namula^{1,2}, Maki Hirata^{1*}, Manita Wittayarat³, Fuminori Tanihara¹, Nhien Thi
11 Nguyen¹, Takayuki Hirano¹, Masahiro Nii⁴, and Takeshige Otoi¹

12 *All correspondence related to this paper should be addressed to: mhirata@tokushima-
13 u.ac.jp

14
15 ¹Faculty of Bioscience and Bioindustry, Tokushima University, Tokushima, Japan;

16 ²College of Agricultural Science, Guangdong Ocean University, Guangdong, China;

17 ³Faculty of Veterinary Science, Prince of Songkla University, Songkhla, Thailand;

18 ⁴Tokushima Prefectural Livestock Research Institute, Tokushima, Japan

19

20

21

22

23

24

25

26

27 **Contents**

28

29 Chlorogenic acid (CGA) and caffeic acid (CA) are potent antioxidants that are mostly
30 found in coffee beans. This study aimed to investigate the effects of CGA and CA
31 supplementation during semen freezing on the quality of frozen-thawed boar spermatozoa.
32 The antioxidants CGA and CA were added to a semen extender to achieve final
33 concentrations of 50, 100, 200 and 400 μM . Supplementation of 100 μM CGA and CA
34 yielded a significantly higher percentage of sperm viability (increased by 8 - 10%) and
35 plasma membrane integrity (increased by 4 - 6%) than the control groups without the
36 antioxidants at 0 h and 3 h after thawing ($P < 0.05$). At a concentration of 100 μM , CGA
37 and CA also yielded beneficial effects on total and progressive sperm motility. Increases
38 of CGA and CA concentrations to more than 200 μM did not enhance any sperm quality
39 parameters. When the sperm penetrability and oocyte development by spermatozoa
40 frozen with CGA and CA were evaluated, CGA and CA supplementations had no positive
41 effects on the percentages of total fertilization, monospermic fertilization, cleavage and
42 blastocyst formation. In conclusion, the supplementation of 100 μM CGA and CA during
43 sperm freezing improved certain sperm parameters including motility, viability and
44 plasma membrane integrity.

45

46

47

48

49 **Keywords:** antioxidants, boar semen, caffeic acid, chlorogenic acid, sperm freezing

50

51

52

53 **1. INTRODUCTION**

54

55 During the process of freezing and thawing, the occurrence of lipid peroxidation
56 caused by the accumulation of reactive oxygen species (ROS) and the removal of natural
57 antioxidants in the seminal plasma could highly damage the lipid tails in the sperm plasma
58 membrane, leading to undesirable effects on spermatozoa functions and their
59 penetrability (Bansal, & Bilaspuri, 2010). Therefore, a balance between ROS production
60 and antioxidant is necessary for sperm stability. Phenolic compounds in coffee beans such
61 as chlorogenic acids (CGA), which is the main class responsible for antioxidant activity,
62 are potent ROS scavengers (Priftis et al., 2018). CGA has *in vitro* free radical scavenging
63 properties and prevents the propagation of oxidative processes (Castro et al., 2018).
64 Caffeic acid (CA) is also found in coffee beans and has a variety of potential
65 immunomodulatory and anti-inflammatory activity in *in vitro* studies and in animal
66 models (Olthof, Hollman, & Katan, 2001). However, their antioxidant action on sperm
67 damage prevention has not yet been elucidated, and it would be interesting to know
68 whether supplementing semen extender with CGA and CA could prevent sperm damage
69 during cryopreservation procedures.

70 This study aimed to examine the effects of CGA and CA supplementation during
71 semen freezing on the quality of frozen-thawed boar spermatozoa, and to evaluate sperm
72 penetrability and oocyte development of spermatozoa frozen with CGA and CA. In the
73 study, therefore, different concentrations of CGA and CA were supplemented to the
74 extender before freezing, and only one concentration of either compound was selected to
75 assess its effects on the penetrability and oocyte development of frozen-thawed
76 spermatozoa.

77

78

79 2. MATERIALS AND METHODS

80

81 All the animals involved in this study received humane care in compliance with the
82 Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory
83 Animal Resources, National Research Council.

84

85 *2.1. Semen collection and cryopreservation*

86 Semen collection and cryopreservation were performed according to the method
87 described by Namula et al. (2014) and Karja et al. (2016) with minor modifications,
88 respectively. Briefly, semen samples were collected once from five fertile Large White
89 boars (2–3 years old) by the gloved-hand technique in November 2017. Semen samples
90 were diluted threefold with Modena extender, and then transported at 25°C to the
91 laboratory within 2 h of collection. The diluted semen was centrifuged at $550 \times g$ for 10
92 min. After removal of the supernatant, each pellet was then diluted to a final concentration
93 of 4×10^8 cells/mL by the first extender supplemented with 100, 200, 400 and 800 μM
94 of CGA (Sigma-Aldrich, St. Louis, MO, USA) or CA (Sigma-Aldrich) at 25°C. The first
95 extender consisted of 0.4 mg/mL D-fructose, 2.9 mg/mL Tris (hydroxymethyl)
96 aminomethane, 1.59 mg/mL citric acid monohydrate, 0.2 mg/mL amikacin sulphate and
97 20% (v/v) egg yolk in distilled water. Conical polystyrene tubes (15 mL) containing the
98 diluted semen samples were placed in a 500-mL glass beaker containing 300–350 mL
99 water at 25°C, which was then kept at 5°C for 2.5 h. After cooling, the second extender
100 (the first extender supplemented with 6% [v/v] glycerol and 1.48% [v/v] EQUEX STM)
101 was added with the same volume of the first extender to achieve the final concentrations
102 of 50, 100, 200 and 400 μM of CGA and CA. The final concentrations of spermatozoa
103 and glycerol were 2×10^8 cells/mL and 3%, respectively. The spermatozoa were loaded
104 into the 0.25-mL French straws and frozen by placing on a Styrofoam plate in liquid

105 nitrogen vapour for 10 min and subsequently plunged into liquid nitrogen. On the day of
106 examination, the straw was immediately submerged into a 38°C water bath for 10 sec for
107 thawing.

108

109 2.2. *Assessment of motility, quality and penetrability of sperm, and oocyte development*

110 Motility analyses were performed using the computer-assisted sperm analysis
111 (CASA) system (Sperm Class Analyzer®: SCA® v.4.2; MICROPTIC, Barcelona, Spain).
112 The analysis of motility was based on the examination of 25 consecutive digitised images
113 obtained from 3–5 fields using a ×10 phase contrast objective, and at least 500
114 spermatozoa per sample were analyzed by the image capture speed with 40 msec.
115 Analyses of the viability, plasma membrane integrity and acrosome integrity were
116 conducted using a live/dead stain combination (SYBR-14/propidium iodide (PI),
117 LIVE/DEAD Sperm Viability Kit; Molecular Probes, Inc., Eugene, OR, USA), the hypo-
118 osmotic swelling test (Ahmad et al., 2003) and fluorescein isothiocyanate-labelled peanut
119 agglutinin (FITC-PNA; Vector Laboratories, Inc., Burlingame, CA, USA), respectively,
120 according to the methods described by Taniguchi et al. (2014). The sperm quality of
121 frozen-thawed spermatozoa was assessed immediately (0 h) and 3 h after thawing of
122 semen.

123 To examine the effects of CGA and CA supplementation during semen freezing on
124 sperm penetrability and oocyte development after *in vitro* fertilization (IVF), cumulus-
125 oocyte complexes matured *in vitro* for 44 h were co-incubated for 20 h with thawed
126 spermatozoa (1×10^6 cells/mL) that had been frozen with either CGA (100 µM) or CA
127 (100 µM) according to a previous method (Do et al., 2015). After co-incubation, some
128 presumptive zygotes were stained with acetic orcein to examine the fertilization of frozen-
129 thawed spermatozoa (Do et al., 2015). The other zygotes were subsequently cultured for

130 7 days to evaluate their ability to develop to blastocysts with a clear blastocoele and cells.

131

132 2.3. *Statistical analysis*

133 The examined parameters were analysed by analysis of variance (ANOVA) using the
134 general linear models (GLM) procedure of SAS (SAS for Windows, version 9.1, SAS
135 Institute, Cary, NC, USA). The data of sperm motility and quality were analysed to assess
136 any effects of treatment, incubation, concentration of antioxidants or an interaction of the
137 two. The differences with a probability value of $P \leq 0.05$ were considered as statistically
138 significant.

139

140 3. RESULTS

141

142 Frozen-thawed spermatozoa treated with 100 μM of CGA and CA yielded
143 significantly higher percentages of viability and plasma membrane integrity than the
144 control groups without CGA and CA ($P < 0.05$) at 0 h and 3 h after thawing (Figs. 1 and
145 2). However, the beneficial effects on viability and plasma membrane integrity were not
146 found when more than 200 μM of CGA and CA were supplemented during semen
147 freezing. Supplementation of CGA and CA did not improve the percentage of
148 spermatozoa with intact acrosomes compared to the control group.

149 At 3 h after thawing, the percentages of total and progressive motility of frozen-
150 thawed spermatozoa treated with 100 μM of CGA and CA were significantly higher than
151 those of the control group ($P < 0.05$) (Figs. 3 and 4). An increase of CGA and CA
152 concentration to more than 200 μM did not enhance the percentages of total and
153 progressive motility. There were no significant differences on the percentages of total
154 fertilization, monospermic fertilization, cleavage and blastocyst formation between
155 treatment and control groups (Table 1).

156

157 **4. DISCUSSION**

158

159 Chlorogenic acid (CGA) is a quinic acid conjugate of caffeic acid (CA) found at high
160 levels in coffee beans and various sources of fruit including strawberries, blueberries,
161 eggplants, and tomatoes (Mahmood et al., 2012). The present study demonstrated that
162 the supplementation of 100 μ M CGA or CA to semen extender had beneficial effects
163 on post-thaw sperm motility, viability and plasma membrane integrity. These results
164 are in agreement with the other reports concerning post-thaw sperm quality have been
165 obtained with, e.g., vitamin E, alpha-tocopherol, glutathione, superoxidase dismutase,
166 and catalase (Grossfeld et al., 2008, Yeste, 2015, Zhang et al., 2012). To be motile,
167 spermatozoa require an adequate supply of energy in the form of ATP produced by
168 mitochondria. Mitochondria is the main site for ROS production, and these ROS can
169 cause harm to mitochondrial DNA (mtDNA), which is highly susceptible to oxidative
170 damage due to its high turnover rate, limited capacity to repair injured DNA and lack
171 of protection by histones (Piomboni et al., 2012). Evgeni et al. (2014) have
172 demonstrated an inverse correlation between DNA fragmentation rate and sperm
173 quality including sperm concentration, motility, viability and morphology. Moreover,
174 it has been reported that cryopreservation can provoke overproduction of ROS that leads
175 to impaired post-thaw sperm motility and morphology (Mazzilli et al., 1995). Therefore,
176 the addition of antioxidants to semen extender might prevent the damage of cryopreserved
177 spermatozoa by ROS and lipid peroxidation toxicity.

178 Coffee phenolics could also protect sperm plasma membrane integrity during
179 cryopreservation, but they had no effects on the fertilization and blastocyst formation,
180 as we have shown in this study. During the first step of fertilization, sperm secrete their
181 acrosomal contents (the 'acrosome reaction') to penetrate the extracellular matrix of the

182 oocyte and reach the oocyte plasma membrane at the site of fertilization (Gadella, &
183 Evans, 2011). The hyper-activation of sperm is critical for the penetration through the
184 zona pellucida of the oocyte (Suarez, & Ho, 2003). In the present study, we observed no
185 significant differences on the percentage of acrosome-intact spermatozoa among the
186 groups at any timepoints. Therefore, one possible reason for no apparent effects of CGA
187 and CA supplementation on the penetrability and oocyte development after IVF could be
188 explained by the high acrosomal integrity maintained in the control group.

189 In conclusion, supplementation of 100 μ M CGA or CA to semen extender has
190 favourable outcomes on post-thaw sperm motility, viability and plasma membrane
191 integrity, but has no effects on acrosome integrity, fertilization and embryonic
192 development in frozen boar spermatozoa.

193

194

195 **5. Acknowledgements**

196

197 This work was supported in part by the Japan Science and Technology Agency/Japan
198 International Cooperation Agency, Science and Technology Research Partnership for
199 Sustainable Development (JST/JICA, SATREPS) and by the Ministry of Education,
200 Culture, Sports, Science and Technology (No.17H03938 and 17K19325).

201

202 **6. Conflict of interest statement**

203

204 None of the authors of this paper has a financial or personal relationship with
205 people or organizations that could inappropriately influence or bias the content of this
206 paper.

207

208 **7. Author contributions**

209

210 Z.N., M.H., and M.W. conceived the study and wrote the manuscript. Z.N.
211 performed most of the experiment and wrote the most part of the manuscript. T.O.
212 designed the study, coordinated all of the experiments and reviewed the manuscript.
213 M.H. participated in the laboratorial work and revised the manuscript. F.T. participated
214 in the laboratorial work and contributed to the statistical analysis. N.T.N. and T.H.
215 participated in the laboratorial work. M.N. collected semen and reviewed manuscript.
216 All authors read and accepted the manuscript.

217

218 **8. References**

219

- 220 Ahmad, Z., Anzar, M., Shahab, M., Ahmad, N., & Andrabi, S. M. (2003). Sephadex and
221 sephadex ion-exchange filtration improves the quality and freezability of low-grade
222 buffalo semen ejaculates. *Theriogenology*, *59*, 1189-1202. 10.1016/S0093-
223 691X(02)01159-7
- 224 Bansal, A. K., & Bilaspuri, G. S. (2010). Impacts of oxidative stress and antioxidants on
225 semen functions. *Veterinary Medicine International*, *2010*. 10.4061/2011/686137
- 226 Castro, A., Oda, F. B., Almeida-Cincotto, M. G. J., Davanco, M. G., Chiari-Andreo, B.
227 G., Cicarelli, R. M. B., Peccinini, R. G., Zocolo, G. J., Ribeiro, P. R. V., Correa,
228 M. A., Isaac, V. L. B., & Santos, A. G. (2018). Green coffee seed residue: A
229 sustainable source of antioxidant compounds. *Food Chemistry*, *246*, 48-57.
230 10.1016/j.foodchem.2017.10.153
- 231 Do, L. T., Luu, V. V., Morita, Y., Taniguchi, M., Nii, M., Peter, A. T., & Otoi, T. (2015).
232 Astaxanthin present in the maturation medium reduces negative effects of heat

233 shock on the developmental competence of porcine oocytes. *Reproductive*
234 *Biology*, 15, 86-93. 10.1016/j.repbio.2015.01.002

235 Evgeni, E., Charalabopoulos, K., & Asimakopoulos, B. (2014). Human sperm DNA
236 fragmentation and its correlation with conventional semen parameters. *Journal of*
237 *Reproduction & Infertility*, 15, 2-14. 10.1111/and.12398

238 Gadella, B. M., & Evans, J. P. (2011). Membrane fusions during mammalian fertilization.
239 *Advances in Experimental Medicine and Biology*, 713, 65-80. 10.1007/978-94-007-
240 0763-4_5

241 Grossfeld, R., Sieg, B., Struckmann, C., Frenzel, A., Maxwell, W. M., & Rath, D. (2008).
242 New aspects of boar semen freezing strategies. *Theriogenology*, 70, 1225-1233.
243 10.1016/j.theriogenology.2008.07.017

244 Karja, N. W., Fahrudin, M., Setiadi, M. A., Tumbelaka, L. I., Sudarwati, R., Hastuti, Y.
245 T., Mulia, B. H., Widiyanti, A., Sultan, K., Terazono, T., Namula, Z., Taniguchi,
246 M., Tanihara, F., Takemoto, T., Kikuchi, K., Sato, Y., & Otoi, T. (2016).
247 Characteristics and Fertility of Sumatran Tiger Spermatozoa Cryopreserved with
248 Different Sugars. *Cryo letters*, 37, 264-271.

249 Mahmood, T., Anwar, F., Abbas, M., & Saari, N. (2012). Effect of maturity on phenolics
250 (phenolic acids and flavonoids) profile of strawberry cultivars and mulberry
251 species from Pakistan. *International Journal of Molecular Sciences*, 13, 4591-
252 4607. 10.3390/ijms13044591

253 Mazzilli, F., Rossi, T., Sabatini, L., Pulcinelli, F. M., Rapone, S., Dondero, F., &
254 Gazzaniga, P. P. (1995). Human sperm cryopreservation and reactive oxygen
255 species (ROS) production. *Acta Europaea Fertilitatis*, 26, 145-148.

256 Namula, Z., Kodama, R., Tanihara, F., Morita, Y., Sato, Y., Wittayarat, M., Taniguchi,
257 M., & Otoi, T. (2014). Effects of skim-milk supplementation on the quality and

258 penetrating ability of boar semen after long-term preservation at 15 degrees C.
259 *Acta Veterinaria Hungarica*, 62, 106-116. 10.1556/AVet.2013.046

260 Olthof, M. R., Hollman, P. C., & Katan, M. B. (2001). Chlorogenic acid and caffeic acid
261 are absorbed in humans. *Journal of Nutrition*, 131, 66-71.

262 Piomboni, P., Focarelli, R., Stendardi, A., Ferramosca, A., & Zara, V. (2012). The role of
263 mitochondria in energy production for human sperm motility. *International*
264 *Journal of Andrology*, 35, 109-124. 10.1111/j.1365-2605.2011.01218.x

265 Priftis, A., Panagiotou, E. M., Lakis, K., Plika, C., Halabalaki, M., Ntasi, G., Veskoukis,
266 A. S., Stagos, D., Skaltsounis, L. A., & Kouretas, D. (2018). Roasted and green
267 coffee extracts show antioxidant and cytotoxic activity in myoblast and
268 endothelial cell lines in a cell specific manner. *Food and Chemical Toxicology*,
269 10.1016/j.fct.2018.02.029

270 Suarez, S. S., & Ho, H. C. (2003). Hyperactivation of mammalian sperm. *Cellular and*
271 *Molecular Biology (Noisy-le-grand)*, 49, 351-356.

272 Taniguchi, M., Wittayarat, M., Morinaga, K., Sato, Y., Kin Do, L. T., Chatdarong, K.,
273 Techakumphu, M., Nii, M., & Otoi, T. (2014). Chelation of trace elements in
274 preservation medium influences the quality of boar spermatozoa during liquid
275 preservation at 5 degrees C for 4 weeks. *Cryo letters*, 35, 336-344.

276 Yeste, M. (2015). Recent Advances in Boar Sperm Cryopreservation: State of the Art and
277 Current Perspectives. *Reproduction in Domestic Animals*, 50 Suppl 2, 71-79.
278 10.1111/rda.12569

279 Zhang, W., Yi, K., Chen, C., Hou, X., & Zhou, X. (2012). Application of antioxidants
280 and centrifugation for cryopreservation of boar spermatozoa. *Animal*
281 *Reproduction Science*, 132, 123-128. 10.1016/j.anireprosci.2012.05.009

282

283 **Figure legends**

284

285 **Figure 1.** Effects of chlorogenic acid (CGA) supplementation during semen freezing on
286 the viability (A), plasma membrane integrity (B) and acrosomal integrity (C) of frozen-
287 thawed spermatozoa. The quality parameters of post-thaw spermatozoa were assessed 0
288 h and 3 h after thawing of semen frozen with various concentration (0–400 μ M) of CGA.
289 The values in one ejaculate from each of five boars were combined to compare the effect
290 of CGA supplementation during semen freezing. ^{a-c}Bars with different letters in the same
291 incubation time differ significantly ($P < 0.05$).

292

293 **Figure 2.** Effects of caffeic acid (CA) supplementation during semen freezing on the
294 viability (A), plasma membrane integrity (B) and acrosomal integrity (C) of frozen-
295 thawed spermatozoa. The quality parameters of post-thaw spermatozoa were assessed 0
296 h and 3 h after thawing of semen frozen with various concentration (0–400 μ M) of CA.
297 The values in one ejaculate from each of five boars were combined to compare the effect
298 of CA supplementation during semen freezing. ^{a-c}Bars with different letters in the same
299 incubation time differ significantly ($P < 0.05$).

300

301 **Figure 3.** Effects of chlorogenic acid (CGA) supplementation during semen freezing on
302 the total motility (A) and progressive motility (B) of frozen-thawed spermatozoa. The
303 sperm motility of post-thaw spermatozoa was assessed 0 h and 3 h after thawing of semen
304 frozen with various concentrations (0 – 400 μ M) of CGA. The values in one ejaculate
305 from each of five boars were combined to compare the effect of CGA supplementation
306 during semen freezing. ^{a-c}Bars with different letters in the same incubation time differ
307 significantly ($P < 0.05$).

308

309 **Figure 4.** Effects of caffeic acid (CA) supplementation during semen freezing on the total
310 motility (A) and progressive motility (B) of frozen-thawed spermatozoa. The sperm
311 motility of post-thaw spermatozoa was assessed 0 h and 3 h after thawing of semen frozen
312 with various concentrations (0 – 400 μ M) of CA. The values in one ejaculate from each
313 of five boars were combined to compare the effect of CA supplementation during semen
314 freezing. ^{a-c}Bars with different letters in the same incubation time differ significantly (P
315 < 0.05).

316

317

318

319

Table 1. Sperm penetrability and oocyte development after in vitro fertilization (IVF) using spermatozoa frozen with chlorogenic acid (CGA) and caffeic acid (CA)

Group	No. of examined oocytes	No. (%) of oocytes fertilized		No. of examined oocytes	No. (%) of embryos	
		Total fertilization	Monospermic fertilization		Cleaved	Developed to blastocysts
Control	282	58.4 ± 2.8	65.5 ± 2.1	392	83.7 ± 2.2	19.8 ± 2.9
CGA	257	62.0 ± 3.6	65.5 ± 2.4	354	82.7 ± 2.4	25.1 ± 3.1
CA	238	64.6 ± 4.6	66.1 ± 1.8	357	82.0 ± 2.4	22.5 ± 2.6

Data expressed as the mean ± SEM. IVF was performed once for each sperm from five boars. Data in the same group was combined to compare the effects of CGA and CA supplementation. Cumulus-oocyte complexes (COCs) were co-incubated with thawed spermatozoa that had been frozen with 100 µM of CGA and CA. As control, the COCs were co-incubated with spermatozoa frozen without CGA and CA. The proportions of monospermic fertilization were calculated by dividing the number of monospermic fertilized oocytes by the total number of fertilized oocytes.

Figure 1

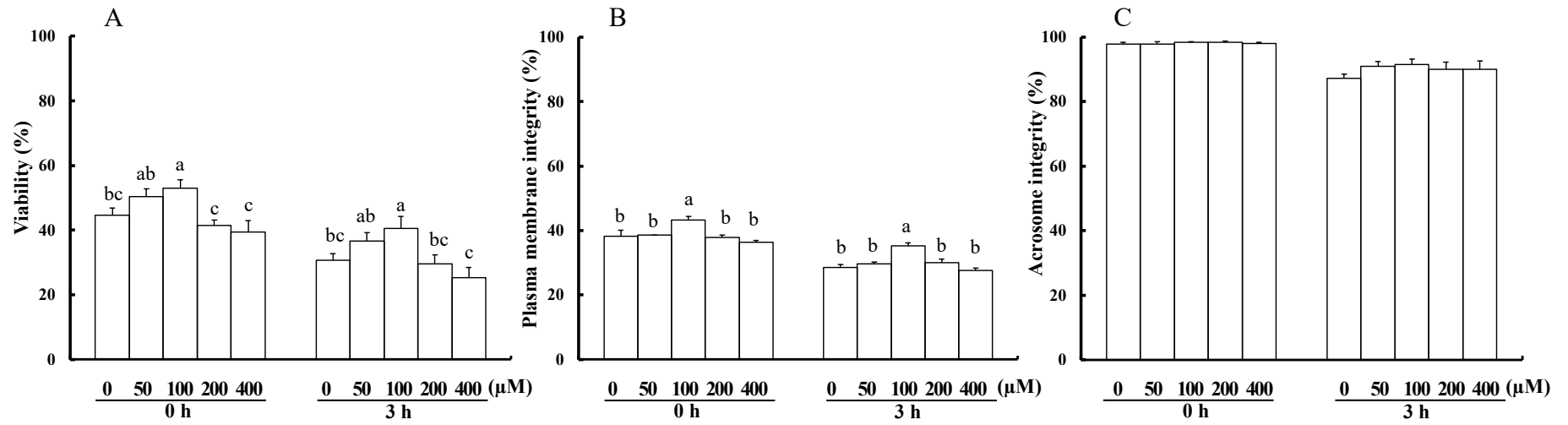


Figure 2

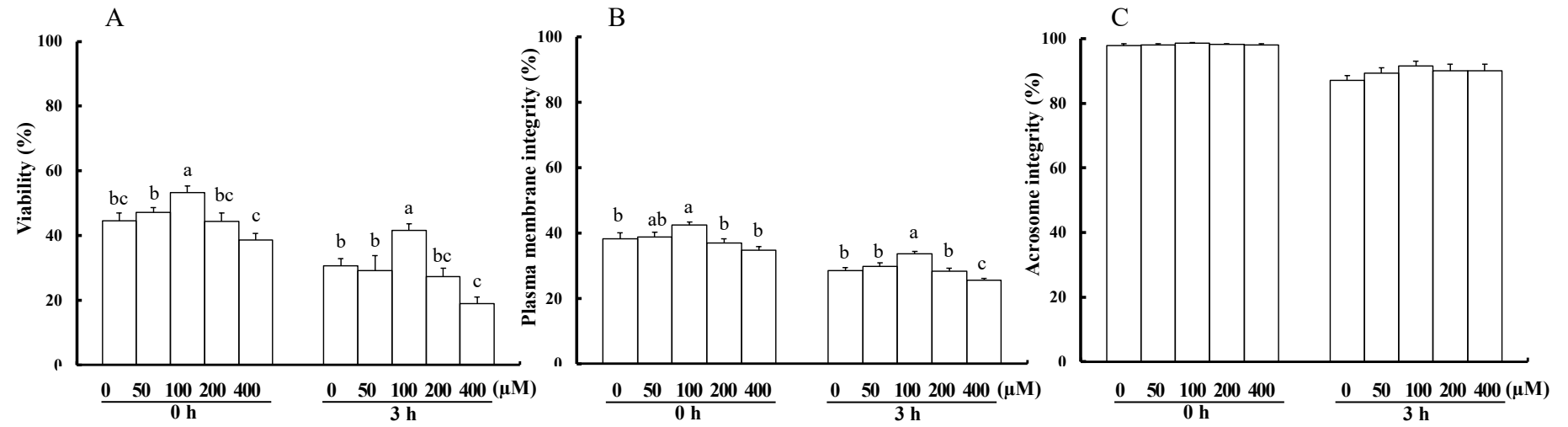


Figure 3

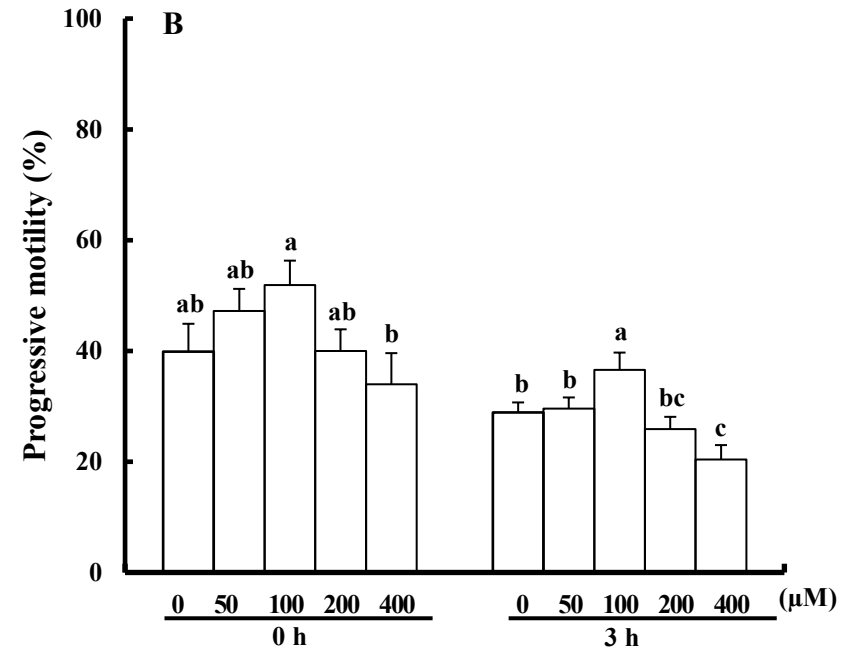
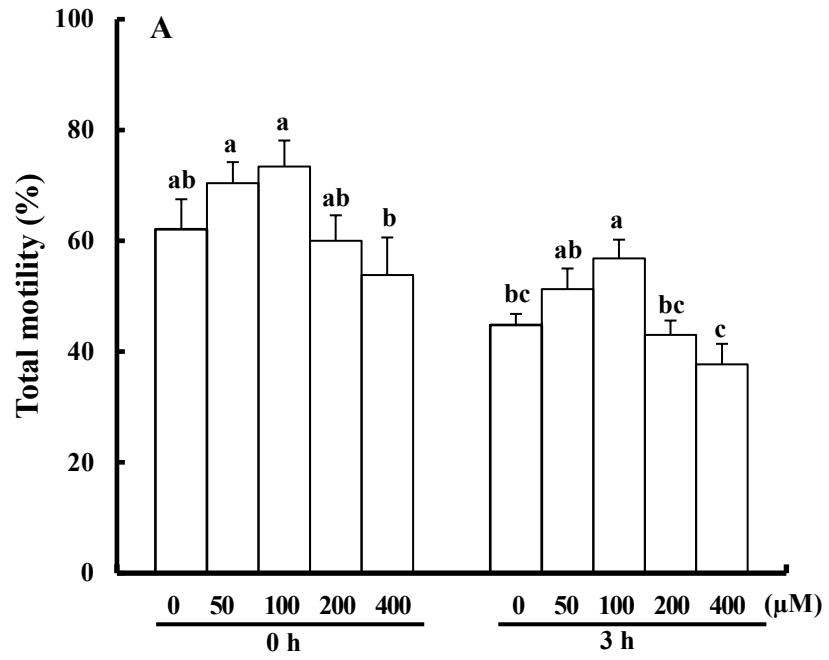


Figure 4

