Title:

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

Running title:

Chlorogenic and caffeic acid supplementation during sperm freezing

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Contents

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

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

1. INTRODUCTION

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

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

2. MATERIALS AND METHODS

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

2.1. Semen collection and cryopreservation

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

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

2.3. Statistical analysis

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

3. RESULTS

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

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

4. DISCUSSION

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

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

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

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

5. Acknowledgements

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6. Conflict of interest statement

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

7. Author contributions

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

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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 μM) 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 μM) 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 μM) 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 ($P < 0.05$).

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

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

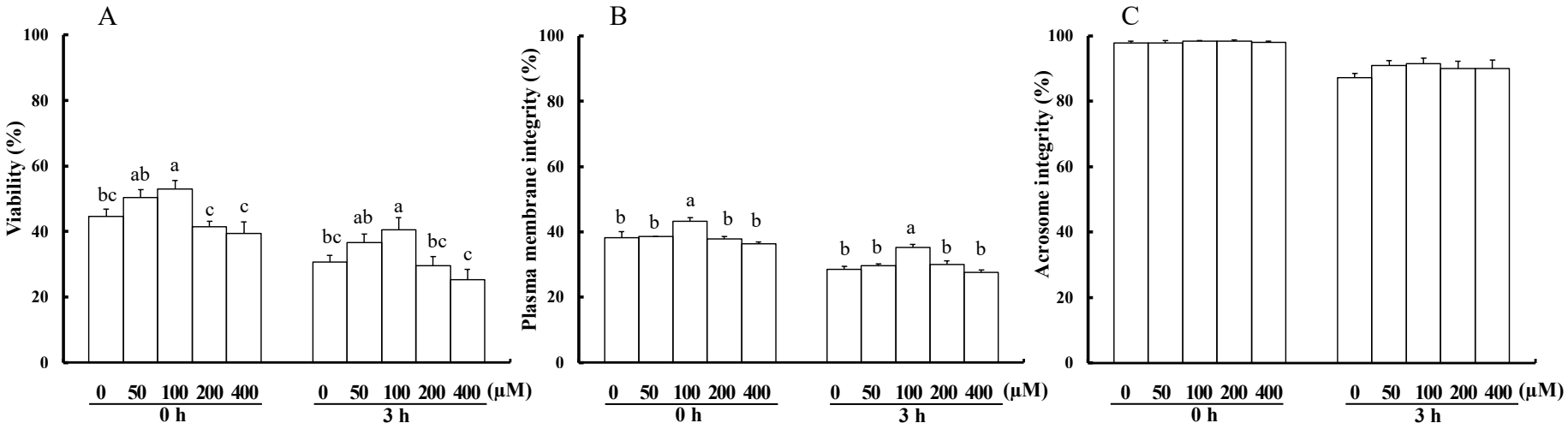
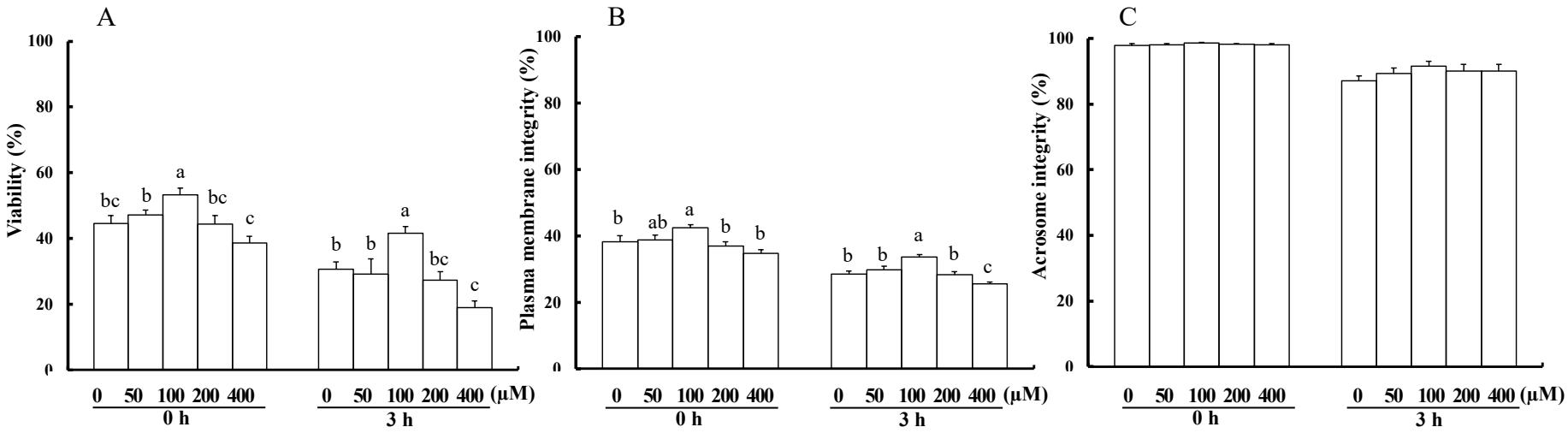

A
B
C
Viability (%)
Plasma membrane integrity (%)
Acrosome integrity (%)
100
80
60
40
20
0
bc ab a c c
bc ab a bc c
b b a b b
b b a b b
0 50 100 200 400 (μM)
0 h
3 h

Figure 2


A
B
C
Viability (%)
Plasma membrane integrity (%)
Acrosome integrity (%)
100
80
60
40
20
0
bc b a bc c
b b a bc c
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b b a b c
0 50 100 200 400 (μM)
0 h
3 h

Figure 3

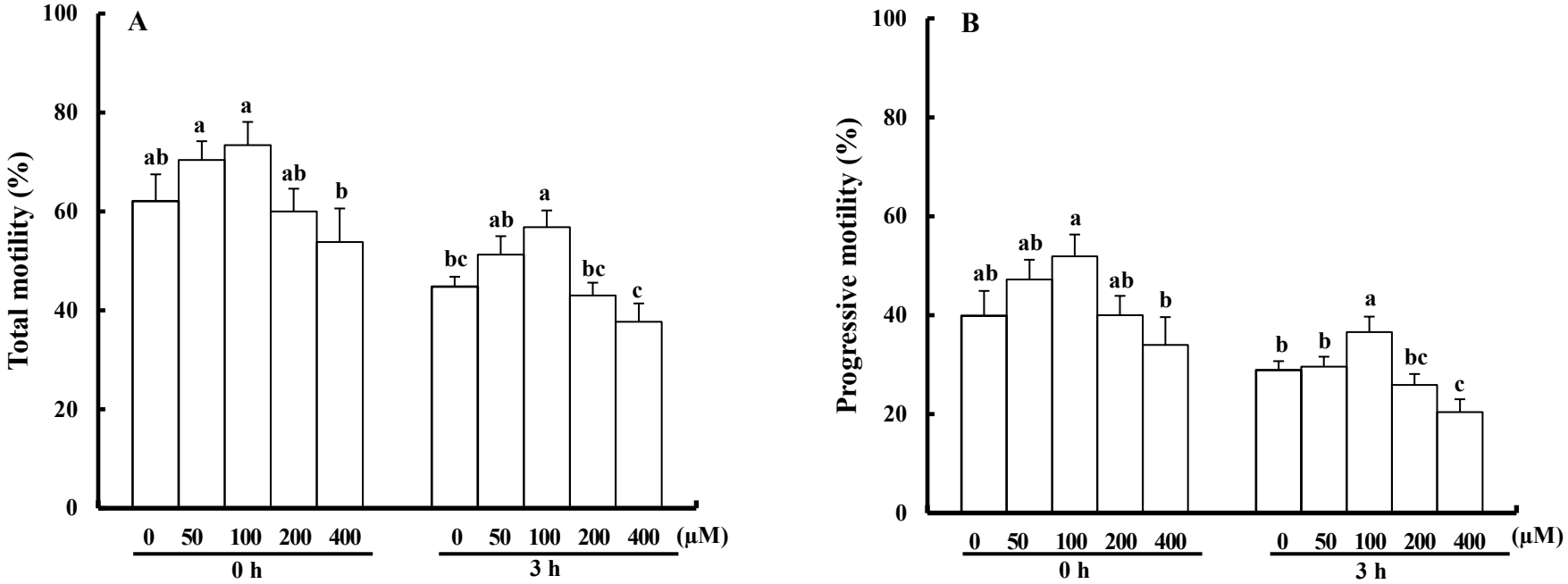
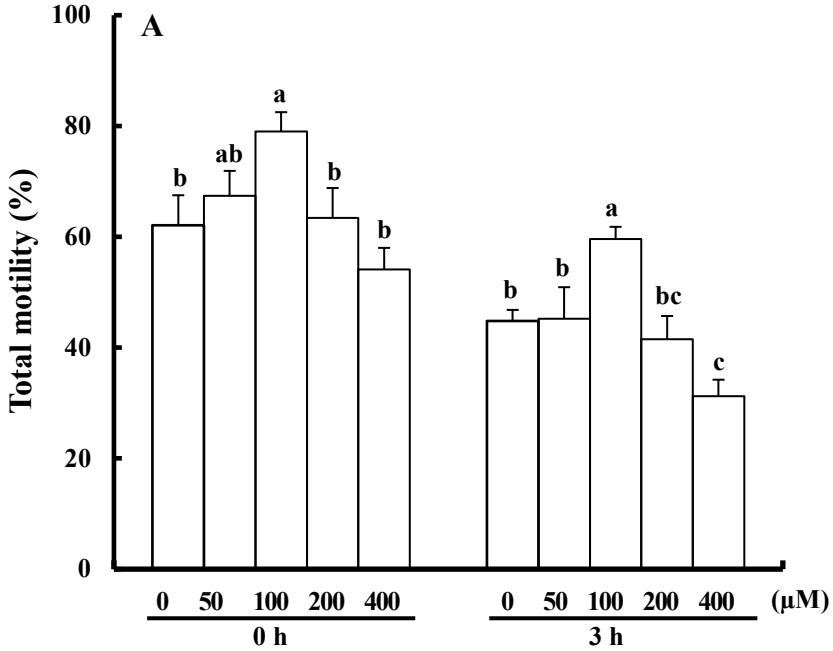
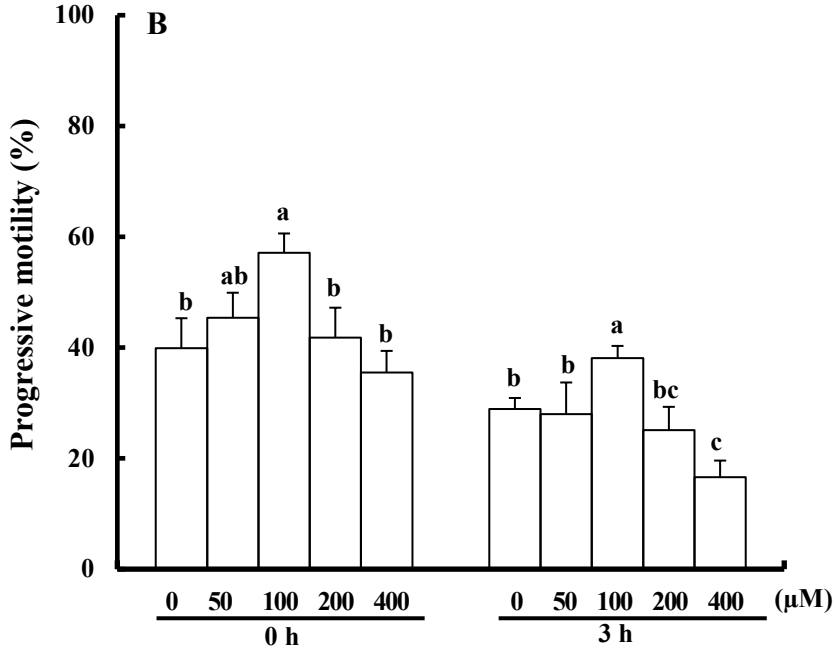

A
Total motility (%)
100
80
60
40
20
0
ab
a
a
ab
b
bc
ab
a
bc
c
0 50 100 200 400
0 50 100 200 400 (μM)
0 h
3 h
B
Progressive motility (%)
100
80
60
40
20
0
ab
ab
a
ab
b
b
b
a
bc
c
0 50 100 200 400
0 50 100 200 400 (μM)
0 h
3 h

Figure 4


A
Total motility (%)
100
80
60
40
20
0
b
ab
a
b
b
b
b
a
bc
c
0 50 100 200 400
0 50 100 200 400 (μM)
0 h
3 h


B
Progressive motility (%)
100
80
60
40
20
0
b
ab
a
b
b
b
b
a
bc
c
0 50 100 200 400
0 50 100 200 400 (μM)
0 h
3 h