Revised

Addition of L-carnitine to the freezing extender improves post-thaw sperm quality of Okinawan native Agu pig

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Abstract: The objective of the present study was to establish whether the addition of L-carnitine (LC), which exhibits antioxidant activity, to the freezing extender improves the quality of cryopreserved Okinawan native Agu pig sperm. Ejaculated sperm frozen in an extender supplemented with 0, 1, 2.5, or 5 mM LC was thawed, and the integrities of mitochondria and the plasmalemma and other sperm characteristics were evaluated. The treatment with different concentrations of LC effectively improved sperm motility, mitochondrial and plasmalemmal integrities, and the proteolytic activity of acrosomal contents after freeze-thawing ($P < 0.05$). The proportion of post-thaw sperm possessing intact mitochondria and plasmalemma and higher proteolytic activity of acrosomal contents was markedly higher among sperm frozen in the presence of 2.5 mM LC than among sperm frozen in the extender without LC ($P < 0.05$). Furthermore, although the addition of LC to the freezing extender had no effect on disturbance of DNA damage and caspase activity, sperm treated with 2.5 mM LC during freezing exhibited significantly higher penetrability into matured oocytes *in vitro* than untreated sperm. Collectively, these results indicate that the addition of LC to the freezing extender effectively improved the post-thaw quality of Agu pig sperm by preventing mitochondrial dysfunction caused by oxidative stress during cryopreservation.

Keywords: Boar spermatozoa, L-carnitine, Cryoinjury, Cell damage, Cryopreservation, Okinawan native Agu pig.

1. Introduction

The long-term preservation and storage of boar sperm for artificial insemination (AI) have become a subject of interest because they allow for the insemination of a large number of females in a short period, avoid the transmission of disease, and conserve valuable germplasm stocks [1]. However, a number of issues are associated with frozen sperm, such as reduced viability, acrosome damage, a short lifespan, cold shock, and membrane damage [2-4]. The cryotolerance of boar sperm is less than that of other domestic animal sperm [5].

One of the deleterious effects that occurs during sperm cryopreservation is oxidative stress elicited by reactive oxygen species (ROS) [6]. While small optimal amounts of ROS are required to maintain normal sperm function, disproportionate levels may have a negative impact on the quality of spermatozoa and impair their overall fertilizing capacity [7]. ROS may also disrupt the membrane structure and function of boar sperm by disordering the membrane phospholipid structure and changing membrane fluidity through lipid peroxidation [8-10]. Therefore, further studies are needed to establish whether the addition of antioxidants to the boar sperm cryopreservation process suppresses the deleterious effects of oxidative stress.

L-carnitine (LC) exhibits antioxidant activity and inhibits lipid peroxidation by transporting fatty acids to the mitochondria for β-oxidation, thereby generating ATP energy [11-13]. LC has been suggested to prevent and/or ameliorate mitochondrial dysfunction caused by a series of conditions, either *in vivo* or *in vitro* [14-16]. Furthermore, LC plays an essential role in mammalian sperm maturation and metabolism because epididymal cells and spermatozoa accumulate energy by increasing ATP generation by LC present in epididymal fluid [17]. Moreover, Lee et al. [18] reported that a treatment with LC exerted positive effects on acrosome reactions and mitochondrial

integrity in the liquid state of fresh semen in miniature pigs. However, cryopreservation processes and treatments with cryoprotectants reduce the intracellular concentration of LC in frozen-thawed spermatozoa. To the best of our knowledge, it has not yet been established whether the addition of LC to the freezing extender preserves boar sperm performance by reducing the detrimental effects of oxidative stress and preventing mitochondrial dysfunction.

The present study was conducted on cryopreserved spermatozoa derived from Okinawan native Agu pig. The reproductive efficiency of Agu has markedly decreased as a consequence of repeated inbreeding within a minority of the closed population [19]. Therefore, the objective of this study was to evaluate the protective effects of the addition of LC to the freezing extender on the post-thawed quality of Agu spermatozoa by measuring motility, the integrities of mitochondria and plasmalemma, the proteolytic activity of acrosomal contents, DNA damage, intracellular caspase activity, and *in vitro* fertilization (IVF) capacity.

2. Materials and methods

All chemicals used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

2.1. Animals

Three clinically healthy Agu boars, aged 1.5 to 3.5 years, were used in the present study. Boars belonging to the corporation limited company Ganaha stock-raising,

92 Okinawa, Japan, were placed in a comfortable environment and maintained on a balanced
93 diet. All protocols were approved by the Institutional Animal Care and Use Committee
94 of the University of the Ryukyus (No. A2020028).
95
96 *2.2. Semen collection and sperm cryopreservation*
97 The sperm-rich fraction of ejaculated semen from three mature Agu (A-1, A-2, and
98 A-3) with proven fertility was collected using the glove-hand technique and filtered
99 through double gauze to remove gel particles. Only samples with more than 70%
100 motile sperm and more than 80% morphologically normal sperm were used in
101 experiments. After collection, semen samples were immediately suspended with an
102 equal volume of Beltsveille thawing solution (BTS; [20]) containing 1% (w/v) skim milk
103 powder and transported at 30°C within 2 h to the laboratory. After semen samples had
104 been centrifuged at 60 × g for 4 min, the supernatants were centrifuged at 1,600 × g for
105 3 min, and seminal plasma was removed by aspiration. After washing in BTS by
106 centrifugation at 1,600 × g at room temperature for 3 min again, sperm sediments were
107 diluted in the Beltsville F5 extender [20] supplemented with 0, 1, 2.5, and 5 mM LC
108 (BF5-LC) at a concentration of 10 $\times 10^8$ sperm/mL, and cooled from 25 to 5°C over a 2-
109 h interval with a cool controlled rate freezer (NCB-3100, EYELA Co., Tokyo, Japan).
110 After standing at 5°C for 1.5 h, the sperm suspension was mixed with the same volume
111 of BF5-LC containing 3% (v/v) glycerol to obtain a final concentration of 5 × 10^8
112 sperm/mL, and 0.1-mL aliquots were quickly frozen on dry ice and stored in liquid
113 nitrogen for more than two weeks.
114
115 *2.3. Analysis of sperm motility*

Frozen-thawed sperm were suspended in 2.5 mL of modified Tyrode solution (114 mM NaCl, 3.2 mM KCl, 0.34 mM NaH_2PO_4, 2 mM $NaHCO_3$, 10 mM HEPES, 0.2 mM Na-pyruvate, 10 mM Na-lactate, 5 mM caffeine, 100 IU/mL penicillin G, and 50 μg/mL streptomycin, pH 7.4) at 39°C. Sperm motility was assessed at 39°C for 3 h using the Sperm Motility Analysis System (SMAS, DITECT, Tokyo, Japan). The proportions of total motile spermatozoa (% TMS, >7 μm/s) and spermatozoa with rapid progressive motility (% RPMS, >50 μm/s) were evaluated by counting a minimum of 200 sperm per sub-sample. These analyses were performed in four replications with different ejaculations.

2.4. Analysis of mitochondrial integrity

Frozen-thawed sperm were diluted in 3 mL Dulbecco's phosphate-buffered saline (PBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 1 mg/mL D-glucose and 1% (w/v) polyvinyl alcohol (PVA) (PBS-PVA). The mitochondrial integrity of frozen-thawed sperm was assessed using dual-color fluorescent staining with Mito Tracker Red CMXRos (Life Technologies, Carlsbad, CA, USA) and SYBR Green I solution (TaKaRa, Bio, Shiga, Japan) as previously described [21]. Briefly, 0.2 μM Mito Tracker Red CMXRos and 0.6 μL SYBR Green I solution were added to the sperm suspension, and sperm were then mixed and incubated at 39°C for 20 min. After centrifugation at 700 × g for 4 min, the sperm sediment was resuspended to a final concentration of 1.0×10^7 sperm/mL in PBS and stored in the dark until analyzed. Sperm showing red fluorescence in the midpiece were considered to be positive for mitochondrial activity under a fluorescence microscope (Nikon, CO., Tokyo, Japan). At least 170 sperm were analyzed per trial in each experimental group, and these analyses

were performed in four replications with different ejaculations.

2.5. Sperm plasmalemma integrity

The plasmalemma integrity of frozen-thawed sperm was assessed using dual-color fluorescent staining with 6-carboxyfluorescein diacetate (10 μg) and propidium iodide (10 μg) as previously described [21, 22]. At least 170 sperm per trial per treatment condition were observed under a fluorescence microscope, and these analyses were carried out in four replications with different ejaculations. Sperm were categorized as having "damaged plasmalemma" when they had partial or complete red fluorescence, or "intact plasmalemma" when they had complete green fluorescence.

2.6. Acrosomal proteolytic activity assay

Acrosomal proteolytic activity in signal sperm was assessed using the gelatin plate assay as previously described [19, 23, 24]. Briefly, 40 μL of 3% (w/v) gelatin suspension was placed across slides and smeared uniformly with another slide by rubbing the two slides together. Slides were fixed for 10 min by immersion in 0.05% (v/v) glutaraldehyde solution and stored vertically in a moist chamber at 4°C until used. Before being used for digestion by spermatozoa, gelatin slides were warmed at room temperature for 5 min.

Frozen-thawed sperm were washed three times by centrifugation at 700 × g for 4 min and resuspended in PBS, giving a 4.0×10^6 sperm/mL concentration. The sperm suspension (20 μL) was placed on one end of a slide and smeared with a cover glass taking care not to disrupt the gelatin film. Slides covered with the gelatin membrane were incubated in a moist chamber at 37°C for 2 h, which led to halo formation. The

horizontal diameters of individual halos around 35 to 45 sperm heads per trial in each experimental group were measured with a micrometer under a phase-contrast microscope and were regarded as an indicator of the proteolytic activity of acrosomal contents. These analyses were performed in four replications with different ejaculations.

2.7. Assessment of DNA damage

Sperm DNA damage was assessed using the neutral comet assay as previously described [19, 21, 25]. Sperm with and without fragmented DNA were indicated by the presence of damaged and intact DNA, respectively, using a fluorescence microscope. At least 80 to 82 sperm were counted per trial in each experimental group, and these analyses were carried out in four replications with different ejaculations.

2.8. Analysis of intracellular caspase activities

Intracellular caspase (caspase-1, -3, -4, -5, -7, -8, and -9) activities in sperm were assessed using the Casp-GLOW Red Active Caspase Staining Kit (Bio Vision, Milpitas, CA, USA). Frozen-thawed sperm were diluted in 8 mL of PBS-PVA, and 300 μL of the sperm suspension containing 1 μL of Red-VAD-FMK solution was incubated at 39°C for 1 h. Sperm were washed twice by centrifugation at 700 × g for 4 min in 500 μL of the assay-wash buffer. The sperm sediment was resuspended to a final concentration of 1.0 × 10^7 sperm/mL in the assay-wash buffer and stored in the dark prior to analysis. Sperm with bright red fluorescence in the midpiece and tail were considered to be positive for caspase activity, based on an assessment with a fluorescence microscope. At least 120 sperm were analyzed per trial in each experimental group, and these analyses were performed in four replications with different ejaculations.

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189 *2.9. Evaluation of IVF capacity*

190 Oocytes from slaughtered adult sows were collected and matured in vitro as
191 previously described [24, 26]. Mature oocytes were stripped of expanded cumulus cells
192 by passage through a narrow-bore pipette in HEPES-buffer Tyrode's medium containing
193 0.01% (w/v) PVA and 0.1% (w/v) hyaluronidase. Oocytes were washed three times with
194 modified Tris-buffered medium (mTBM) designated as IVF medium and supplemented
195 with 2 mM caffeine sodium benzoate and 3 mg/mL BSA. Groups of 25 to 30 oocytes
196 were finally transferred to 50-μL droplets of IVF medium that had been covered with
197 warm mineral oil. Droplets were kept in an incubator under 5% CO_2 at 39°C for 1 h
198 until sperm were added for fertilization.

199 After thawing and washing, pellets were resuspended at 4×10^8 sperm/mL in
200 mTBM supplemented with 4 mM caffeine sodium benzoate and 4 mg/mL BSA, and then
201 incubated at 39°C for 90 min in a 5% CO_2 incubator. After the preincubation, 50 μL of
202 the diluted sperm suspension in IVF medium was added to a droplet containing oocytes
203 at a final concentration of 1×10^6 sperm/mL. Gametes were co-incubated for 10 h under
204 the conditions indicated above. Accessory sperm were removed by gentle vortexing at
205 the end of the period. Oocytes were mounted, fixed for 72 h in 25% (v/v) acetic acid in
206 ethanol, stained with 1% (w/v) lacmoid in 45% (v/v) acetic acid, and examined under a
207 phase-contrast microscope at × 400 magnification. Oocytes were designated as
208 penetrated when one or more swollen sperm heads and/or male pronuclei and
209 corresponding sperm trails were present. These analyses were achieved in four
210 replications with different ejaculations.

211

212 *2.10. Experimental design*

213 Experiment 1 examined whether sperm motility, mitochondria and plasmalemma
214 integrities, the proteolytic activity of acrosomal contents, DNA damage, and intercellular
215 caspase activity were improved by the treatment with LC during the freezing procedure.
216 Each ejaculated sperm sample was split into four aliquots and extended with BF5
217 supplemented with 0, 1, 2.5, and 5 mM LC. Extended aliquots were cryopreserved
218 using previously described procedures.

219 Experiment 2 was designed to further elucidate the effects of the addition of LC to
220 the freezing extender on IVF capacity. From the findings in Experiment 1, 2.5 mM of
221 LC added to the freezing extender was optimum for preventing cryoinjury in boar sperm.
222 Therefore, each ejaculated sperm sample was split into two aliquots and cryopreserved in
223 BF5 supplemented with LC at a final concentration of 0 (as a control) or 2.5 mM. After
224 thawing, the ability to fertilize matured oocytes in vitro was measured.

225

226 *2.11. Statistical analysis*

227 Data from four ejaculate trails for each boar were expressed as means ± SEM. All
228 statistical analyses were performed using the Statistical Analysis System R software
229 package (http:/www.R-project.org/). To evaluate differences between LC treatment
230 groups, percentage data were analyzed using the generalized linear model (GLM,
231 according to a binomial distribution) and ANOVA procedures followed by Ryan's test for
232 non-parametric multiple companions [27]. Analyses of other data (halo diameter, and
233 the number of sperm per penetrated oocyte) were performed with the Shapiro-Wilk
234 normality test and GLM (according to the Gaussian distribution) and ANOVA procedures
235 followed by the Tukey-Kramer test. Significant differences in post-hoc test ($P < 0.05$)

among the main factors and interactions were also examined. The chi-squared test evaluated data for fertilization parameters in individual boars. A probability of $P < 0.05$ was considered to be significant.

3. Results

In experiment 1, no significant interaction was observed between treatments with LC and the ejaculate for any data on post-thaw sperm characteristics. The effects of the treatment with LC during the freezing procedure on post-thaw sperm motility parameters (TMS and RPMS) are shown in Tables 1 and 2. The percentage of TMS 3 h after thawing was higher among sperm treated with 2.5 mM LC during the freezing procedure than among untreated sperm in all individuals ($P < 0.05$). Correspondingly, RPMS motility slightly increased in post-thaw sperm frozen in the extender containing 2.5 mM LC.

The freezing extender supplemented with 2.5 mM LC increased the incidence of post-thaw sperm possessing intact mitochondria in all individuals over that at other concentrations of the LC treatment ($P < 0.05$) (Fig. 1A). Similarly, plasmalemma integrity was markedly ($P < 0.05$) increased by the treatment with LC at a concentration of 2.5 mM in all individuals (Fig. 1B). The percentage of sperm with an intact plasmalemma in A-1 and A-3 was higher at 2.5 mM LC than at the other concentrations tested ($P < 0.05$). Similarly, the halo diameter as an indicator of the proteolytic activity of acrosomal contents was increased in post-thaw sperm treated with 2.5 mM LC during the freezing procedure in all individuals ($P < 0.05$) (Fig. 1C). Mitochondrial and

plasmalemma integrities and the proteolytic activity of acrosomal contents showed an interaction between treatment and boars ($P < 0.05$).

However, the incidence of post-thaw sperm possessing intact DNA and activated caspases in all individual sperm did not show any significant changes during cryopreservation regardless of the treatment with LC (Fig. 2).

In Experiment 2, the treatment with 2.5 mM LC during the freezing procedure preserved *in vitro* sperm penetrability in post-thaw sperm in all individuals ($P < 0.05$), and the interaction between the LC treatment and individual boars was not significant (Table 3). Besides, LC treatment in the extender had no effect on polyspermy and male pronucleus formation rates or the mean number of sperm per oocyte.

4. Discussion

Various cryoprotective media have been designed in an attempt to improve the quality of post-thaw boar sperm [19, 28-31]. The present study demonstrated that a treatment with LC during cooling and freezing protected Agu sperm against damage due to cold shock, thereby improving post-thaw sperm characteristics over those of sperm frozen in the extender without LC.

The addition of LC to the freezing extender was recently shown to improve the chances of preserving the characteristics of sperm in buffalo [32] and humans [33]. In the present study, sperm motility after thawing was maintained at a high quality in sperm frozen in the extender containing 2.5 mM LC (Tables 1 and 2). The decline observed in the motility of human sperm incubated with ROS may be attributed to ATP depletion

[34]. LC positively provides ATP energy by transporting fatty acids to the mitochondria for β-oxidation [35]. Post-thaw sperm motility was previously shown to be enhanced in cryopreserved buffalo sperm due to LC-mediated improvements in mitochondria function and ATP generation [32]. In the present study, mitochondrial and plasmalemmal integrities as well as the halo diameter as an index for acrosome integrity were markedly higher in all post-thaw Agu sperm treated with 2.5 mM LC than in sperm not treated with LC (Fig. 1). LC reduces the availability of lipids for peroxidation and suppresses mitochondrial dysfunction by transporting activated fatty acids across the inner membrane of mitochondria for β-oxidation to generate ATP energy [11, 12, 14-16, 21, 35]. On the other hand, lipid peroxidation induces membrane damage [36]. Furthermore, Jones and Mann [37] reported that cell membrane damage caused by lipid peroxidation was prominent as damage to the acrosome site. Therefore, the function of LC to transport fatty acids into mitochondria appeared to suppress mitochondrial dysfunction and lipid peroxidation, resulting in the strong protection of mitochondria, the plasmalemma, and acrosome during the freezing procedure of boar sperm. The present results infer that 2.5 mM of LC added to the freezing extender is optimal for supporting sperm motility, and the integrities of mitochondria, plasmalemma and acrosome in post-thaw boar sperm. However, the treatment with a higher LC concentration (5 mM) did not result in further improvements; it actually reduced mitochondrial and plasmalemma integrities and the proteolytic activity of acrosomal contents. Higher concentrations of LC may function as an oxidation stimulator rather than as an antioxidant, similar to ascorbic acid [38]. Higher concentrations of LC were previously shown to be toxic in cat epididymis sperm [39], and increased the fluidity of the plasma membrane above the desired point, making sperm more susceptible to lipid

peroxidation [40].

In the present study, the treatment with LC during the sperm cooling and freezing procedure did not prevent DNA damage or inhibit caspase activity (Fig. 2). Similar findings were reported for human sperm, for which a treatment with LC did not suppress DNA fragmentation during cryopreservation [33]. DNA fragmentation correlated with apoptotic-cell death, decreased semen quality, and an impaired potential for fertilization [41]. Caspases generally cause DNA fragmentation due to apoptosis [42]. Excessive amounts of ROS induce the activation of caspase-8, which is located downstream of Fas (also known as CD95 or APO-1), and are a key mediator of apoptotic signals associated with death receptors [43-46]. In our recent study, the O_2^--scavenging activity of LC markedly increased in a concentration-dependent manner, whereas its radical-scavenging activity was very low (unpublished data). Gulicin [13] reported that the antioxidant activity of LC may be attributed to its ability to chelate free ferrous ions, inhibit generated O_2^-, and detoxify accumulated hydrogen peroxide species. Based on these findings, the treatment with LC did not prevent DNA damage or inhibit caspase activity during the freezing procedure due to insufficient protection against oxidative stress caused by free radicals, the production of which increases during sperm cryopreservation [47].

The acrosomal status of frozen-thawed boar sperm is an important indicator of fertilization ability [48]. Furthermore, the RPMS parameter of post-thaw sperm correlated with *in vivo* fertility in boars [49]. The present results showed that an optimal concentration of LC increased the motility and prevented damage to the mitochondria, plasmalemma, and acrosome of frozen-thawed sperm, resulting in the significant preservation of *in vitro* sperm penetrability in all individuals (Table. 3). Based on these results, after the AI of commercial sows (Kenboro) using Agu sperm frozen in the

presence of 2.5 mM LC during the winter season, the conception rate (80%; 20/25) and farrowing rate (68%; 17/25) were prominently higher than those obtained by using the fresh semen (54%; 55/101, and 50%; 51/101, respectively) during the extremely hot season between July and September (unpublished observations).

In conclusion, the present study demonstrated that a treatment with LC at a certain concentration (2.5 mM) during cooling and freezing efficiently enhanced sperm motility, and maintained mitochondrial, plasmalemmal, and acrosomal integrities, and *in vitro* sperm penetrability, but did not obviate DNA damage or caspase activity in post-thaw Agu sperm. In further studies, other antioxidants that exhibit radical-scavenging activity need to be used with LC in order to take advantage of this technique for sperm cryopreservation in commercial pig breeds and establish safer AI or IVF treatments to support the reproductive efficiency of the Agu pig.

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520 **Figure Legends**

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522 Fig. 1. Effects of various concentrations of L-carnitine (LC) added to the freezing
523 extender on mitochondrial integrity (A), plasmalemma integrity (B), and halo diameter
524 (C) in post-thaw Agu sperm. Values are expressed as the mean ± SEM of four ejaculates
525 from three Agu. The total numbers of sperm examined were 720 to 726 (A), 720 to 740
526 (B), and 103 to 112 (C) in each treatment group. [a-d] Significant differences were
527 observed within the LC treatment groups in the same pigs as assessed by a one-way
528 ANOVA ($P < 0.05$). [A-C] Significant differences were observed among individuals as
529 assessed by GLM-ANOVA procedures ($P < 0.05$).

530

531 Fig. 2. Effects of various concentrations of L-carnitine added to the freezing extender
532 on DNA integrity (A) and intercellular caspase activity (B) in frozen-thawed Agu sperm.
533 Values are expressed as the mean ± SEM of four ejaculates from three Agu. The total
534 numbers of sperm examined were 328 to 330 (A) and 701 to 779 (B) in each treatment
535 group. [a-c] Significant differences were observed within the LC treatment groups in the
536 same pigs as assessed by a one-way ANOVA ($P < 0.05$). [A, B] Significant differences
537 were observed among individuals as assessed by GLM-ANOVA procedures ($P < 0.05$).

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540 Table 1. Total motile spermatozoa (TMS) after freeze-thawing with various
541 concentrations of L-carnitine (LC).

Agu	Concentration of L-carnitine (mM)	Incubation time after thawing (h)		
		0	1	3
A-1 [A]	0	84.9 ± 1.0 [a, x]	73.4 ± 1.4 [a, y]	22.4 ± 1.4 [a, z]
	1	81.1 ± 1.2 [a, x]	87.6 ± 0.9 [b, x]	34.4 ± 1.5 [b, y]
	2.5	87.4 ± 0.9 [a, x]	85.4 ± 1.0 [b, x]	41.7 ± 1.6 [c, y]
	5	68.0 ± 1.5 [c, x]	76.4 ± 1.3 [a, y]	39.6 ± 1.4 [c, z]
A-2 [B]	0	64.5 ± 1.7 [a, x]	62.8 ± 1.7 [a, x]	24.3 ± 1.9 [a, y]
	1	64.6 ± 1.6 [a, x]	66.3 ± 1.5 [a, x]	32.8 ± 1.5 [b, y]
	2.5	69.4 ± 1.6 [a, x]	67.4 ± 1.6 [a, x]	38.2 ± 1.6 [c, y]
	5	67.2 ± 1.6 [a, x]	63.2 ± 1.5 [a, x]	43.9 ± 1.7 [c, y]
A-3 [C]	0	82.8 ± 1.1 [a, x]	82.6 ± 1.4 [a, x]	24.9 ± 1.4 [a, y]
	1	81.1 ± 1.2 [a, x]	72.1 ± 1.5 [bc, y]	29.1 ± 1.6 [ab, z]
	2.5	83.3 ± 1.3 [a, x]	78.4 ± 1.3 [ac, y]	33.4 ± 1.6 [b, z]
	5	81.7 ± 1.2 [a, x]	69.6 ± 1.5 [b, y]	25.8 ± 1.6 [a, z]

542 Values are expressed as the mean ± SEM of four ejaculates from three Agu.
543 [a-c] Values with different superscripts in the same column are significantly different
544 within the LC treatment groups in the same individuals ($P < 0.05$).
545 [x-z] Values with different superscripts in the same row are significantly different ($P <$
546 0.05).
547 [A-C] Values with different superscripts are significantly different among individuals ($P <$
548 0.05).
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556 Table 2. Rapid progressive motility spermatozoa (RPMS) after freeze-thawing with
557 various concentrations of L-carnitine (LC).

Agu	Concentration of L-carnitine (mM)	Incubation time after thawing (h)		
		0	1	3
A-1 [A]	0	12.5 ± 0.9 [ac, x]	13.9 ± 1.1 [a, y]	0.7 ± 0.3 [ab, z]
	1	17.0 ± 1.2 [b, x]	12.5 ± 0.9 [a, y]	1.6 ± 0.4 [bc, z]
	2.5	10.0 ± 0.8 [a, x]	11.3 ± 0.9 [a, x]	2.9 ± 0.6 [c, y]
	5	14.4 ± 1.1 [c, x]	13.5 ± 1.0 [a, x]	1.7 ± 0.4 [bc, y]
A-2 [A]	0	9.8 ± 1.1 [ab ,x]	15.1 ± 1.3 [a, y]	1.8 ± 0.6 [a, z]
	1	8.9 ± 1.0 [ab, x]	9.5 ± 1.0 [b, x]	1.8 ± 0.4 [a, y]
	2.5	16.6 ± 1.3 [c, x]	17.0 ± 1.3 [a, x]	3.0 ± 0.6 [ab, y]
	5	6.4 ± 0.8 [a, x]	10.0 ± 0.9 [b, y]	4.7 ± 0.7 [b, y]
A-3 [B]	0	19.8 ± 1.2 [a, x]	16.9 ± 1.3 [a, x]	0.7 ± 0.3 [a, y]
	1	17.0 ± 1.2 [a, x]	21.0 ± 1.4 [b, x]	1.7 ± 0.5 [a, y]
	2.5	18.5 ± 1.4 [a, x]	15.0 ± 1.1 [a, x]	1.6 ± 0.4 [a, y]
	5	26.7 ± 1.4 [b, x]	14.9 ± 1.2 [a, y]	0.7 ± 0.3 [a, z]

558 Values are expressed as the mean ± SEM of four ejaculates from three Agu.
559 [a-c] Values with different superscripts in the same column are significantly different
560 within the LC treatment groups in the same individuals ($P < 0.05$).
561 [x-z] Values with different superscripts in the same row line are significantly different (P
562 < 0.05).
563 [A, B] Values with different superscripts are significantly different among individuals (P
564 < 0.05).
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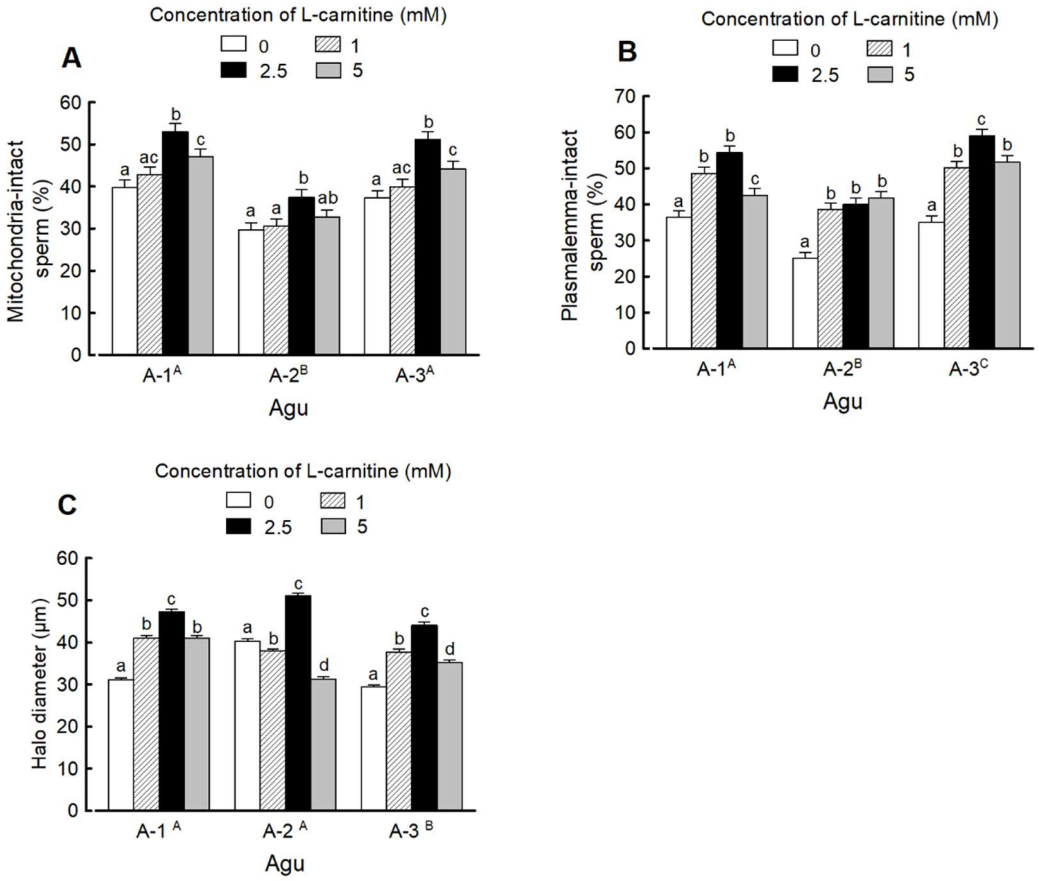

Fig. 1. Effects of various concentrations of L-carnitine (LC) added to the freezing extender on mitochondrial integrity (A), plasmalemma integrity (B), and halo diameter (C) in post-thaw Agu sperm. Values are expressed as the mean ± SEM of four ejaculates from three Agu. The total numbers of sperm examined were 720 to 726 (A), 720 to 740 (B), and 103 to 112 (C) in each treatment group. [a-d] Significant differences were observed within the LC treatment groups in the same pigs as assessed by a one-way ANOVA ($P < 0.05$). [A-C] Significant differences were observed among individuals as assessed by GLM-ANOVA procedures ($P < 0.05$).

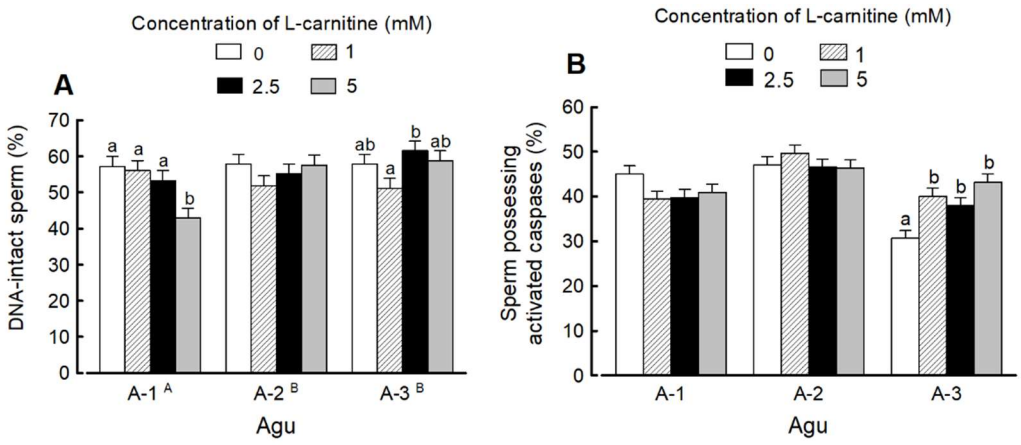

Fig. 2. Effects of various concentrations of L-carnitine added to the freezing extender on DNA integrity (A) and intercellular caspase activity (B) in frozen-thawed Agu sperm. Values are expressed as the mean ± SEM of four ejaculates from three Agu. The total numbers of sperm examined were 328 to 330 (A) and 701 to 779 (B) in each treatment group. [a-c] Significant differences were observed within the LC treatment groups in the same pigs as assessed by a one-way ANOVA ($P < 0.05$). [A, B] Significant differences were observed among individuals as assessed by GLM-ANOVA procedures ($P < 0.05$).

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597 Table 3. Effects of L-carnitine added to the freezing extender on fertilization parameters
598 in post-thaw Agu sperm.

Agu	L-carnitine (mM)	No. of oocytes examined	Oocytes (%; mean ± SEM)			No. of sperm per penetrated oocyte (mean ± SEM)
			Penetrated	Polyspermic[1)]	Male pronucleus [1)]	
A-1	0	151	28.5 ± 3.7	34.9 ± 7.3	72.1 ± 6.8	1.4 ± 0.0
	2.5	173	48.0 ± 3.8 *	39.0 ± 5.4	82.9 ± 4.2	1.5 ± 0.0
A-2	0	143	39.2 ± 4.1	30.6 ± 6.4	57.1 ± 6.6	1.3 ± 0.2
	2.5	128	55.1 ± 4.4 *	30.0 ± 4.7	68.6 ± 5.5	1.4 ± 0.1
A-3	0	116	36.2 ± 4.5	42.9 ± 7.6	66.7 ± 7.3	1.6 ± 0.1
	2. 5	137	54.0 ± 4.3 *	31.1 ± 5.4	66.2 ± 5.5	1.5 ± 0.2

599 [1)] Percentage of oocytes that were penetrated.

600 * Values are significantly different between two treatments in each individual ($P < 0.05$).

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