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L-Carnitine in rooster semen cryopreservation: Flow cytometric, biochemical and motion findings for frozen-thawed sperm

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ABSTRACT

Rooster semen cryopreservation is not efficient for artificial insemination in breeder flocks. L-Carnitine (LC) has been evaluated for effectiveness in cryopreservation media on the characteristics of rooster sperm after freeze-thawing. Motility characteristics, membrane functionality, abnormal morphology, apoptotic like changes, mitochondria activity and lipid peroxidation of rooster sperms were assessed after freeze-thawing with different concentrations of LC in Beltsville medium. Semen samples were collected from 12 roosters, twice a week, and diluted in the extenders that contained different concentrations of LC. Supplementation of Beltsevile with 1 and 2 mM LC was found to result in higher total motility (68.2± 1.7% and 69.1± 1.7%, respectively), progressive motility (28.4± 1.6%, 29.8± 1.6%), membrane functionality (76.2± 1.9% and 75.9± 1.9%), viability (58.2 ± 1.1%, 59.1 ± 1.1%) and lower significant of lipid peroxidation (2.53 ± 0.08 nmol/ml, 2.49 ± 0.08 nmol/ml) compared to control group containing no LC. Lower motility, progressive motility, and viability were observed in frozen-thawed sperm in extender containing 8 mM LC (35.8± 1.7%, 9.6± 1.2% and 27.1 ± 1.2%, respectively) compared to control. Morphology and mitochondrial activity were not affected by different concentrations of LC. Our results showed that supplementation of Beltsville extender with 1 and 2 mM LC significantly improved the quality of rooster sperm quality after freeze-thawing.

1. Introduction

Cell membrane of avian sperm contains high amounts of polyunsaturated fatty acids (PUFAs) that can easily undergo lipid peroxidation (LPO) in the presence of reactive oxygen species (ROS) [11,44,50]. ROS are reactive molecules with destructive effects on sperm plasma membrane, which lead to reduce function and freezing ability of sperm [1,24,25,30]. Although rooster sperm possesses antioxidant systems such as glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) [27,40], their activities may be affected by the freeze-thawing process [27,40,48]. Therefore, providing an auxiliary protection system is crucial for rooster sperm integrity after freeze-thawing. L-Carnitine (LC) is a water-soluble amino acid that is naturally produced in the animal body [6]. It has crucial roles in generation of metabolic energy by facilitating transportation of fatty acids into mitochondria [16]. Moreover, antioxidant characteristics and anti-apoptotic activities of LC may stabilize mitochondrial membrane and DNA structure against ROS [32]. LC also increases the activity and levels of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase [29]. Menchini-Fabris et al. (1984), observed a positive correlation between free LC and sperm quality in human semen [26]. Supplementation of human sperm freezing medium with LC prior to cryopreservation has been found to increase the motility [49], acrosome integrity [4] and morphology [8] of human sperm after cryopreservation. In animal species, LC has been found to significantly improve the motility of rainbow trout [19] and bull [35] sperm. There have also been reports of beneficial effects of dietary LC on the fertility and sperm quality of boar [18,46], human [45], chicken [29] and quails [34]. To our knowledge, there have been no studies yet, that have the roles of LC in cryopreservation of

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rooster semen. In this work, the freezing medium was supplemented with different concentrations of LC to determine the effects of LC on motion characteristics, morphology, membrane functionality, mitochondrial activity, apoptotic-like changes and lipid peroxidation of sperm after freeze-thawing.

2. Materials and methods

2.1. Chemicals

All chemicals used in this study were purchased from Sigma (St. Louis, MO, USA), and Merck (Darmstadt, Germany). The approval for the present study was given by the Research Ethics Committees of Royan Institute.

2.2. Farm management and semen collection

This experiment was conducted on Ross broiler breeders comprising 12 adult males (32 weeks) kept individually in cages (70 × 60 × 75 cm) at 18–22 °C, under 15 L: 09 D photoperiod.

Sperm collection was carried out two times a week using abdominal massage [10] and then transferred to a semen collection tube. After collection, the sperm was placed in a water bath (37 °C), for primary evaluation in laboratory. The criteria for normal quality of sperm consisted of: volume: 0.2–0.6 ml; sperm concentration of $\geq 3 \times 10^9$ spermatozoa/ml; motility ≥80% and abnormal morphology ≤10%. Then, to eliminate individual differences, semen samples were pooled and divided into six aliquots according to the experimental design.

2.3. Extender preparation and cryopreservation

The components of basic freezing medium (Beltsville) used in this study were dipotassium phosphate (43 mM) sodium glutamate (51 mM), fructose (27 mM), sodium acetate (39 mM), TES [n-tris (hydroxymethyl) methyl 1–2 amino ethane sulfonic acid] (13 mM), monopotassium phosphate (5.1 mM), potassium acetate (6.5 mM), magnesium chloride (3.7 mM) [38]. Glycerol was added to the basic medium at 3% (v/v) with pH of 7.1 and osmolarity of 310 mOsm/kg. The following samples were made for the study: Beltsville without LC (control), Beltsville with 0.5 mM (L0.5), 1 mM (L1), 2 mM (L2), 4 mM (L4) and 8 mM (L8) LC. Sperm samples were diluted with freezing media in their groups and then aspirated into 0.25 ml French straws (IMV, L'Aigle, France) to obtain final concentration of 400×10^6 sperm/ml. Then straws were sealed with polyvinyl alcohol powder and equilibrated at 5 °C for a period of 3 h. After equilibration, the straws were frozen in liquid nitrogen vapor, 4 cm above the liquid nitrogen for 7 min in a 40 × 20 × 20 cm cryobox containing 8000 cm^3 liquid nitrogen. Then, the straws were plunged into liquid nitrogen for storage. The frozen straws were then thawed individually at 37 °C for 30 s in a water bath for evaluation.

2.4. Motion parameters

Sperm class analysis software (Version 5.1; Microptic, Barcelona, Spain) was used to analyze sperm motion characteristics. For this purpose, thawed sperm samples were first diluted to 20×10^6 sperm/mL with PBS buffer. Then, 5 ml samples of diluted semen were placed onto a prewarmed chamber slide (38 °C, Leja 4; 20 mm height; Leja Products, Luzernestraat B.V., Holland), and sperm motility characteristics were determined [20]. At least six fields that contained a minimum of 400 sperm were evaluated for each sample at a 5-s average time to read each sample. The following values were recorded: motility (%), progressive motility (%), average path velocity (in micrometers per second), straight linear velocity (in micrometers per second), curvilinear velocity (in micrometers per second), and amplitude of lateral head displacement (in micrometers).

2.5. Membrane functionality

Hypoosmotic swelling test (HOST) was used to evaluate the functionality of sperm plasma membrane after freeze-thawing [33]. HOST relies on the resistance of the sperm's membrane to stressful conditions in a hypoosmotic medium. This assay was carried out by mixing 5 μl of semen with a 50 μl hypoosmotic solution (100 mOsm/l, 57.6 mM fructose and 19.2 mM sodium citrate). After 20 min incubation, the sperm was checked under a phase-contrast microscope (CKX41, Olympus, Tokyo, Japan) and 300 sperm with swollen and non-swollen tails were recorded.

2.6. Sperm morphology

For evaluation of total abnormalities, three drops (10 μl) of semen were pipetted into 1 ml Hancock's solution [37]. Hancock's solution consisted of 62.5 ml formalin (37% formaldehyde), 150 ml of sodium saline solution, 150 ml of PBS buffer solution and 500 mL of double-distilled water. To detect abnormality of sperm acrosome, head and tail, 10 ml of processed sperm was handled on a slide. The percentage of sperm abnormalities was recorded by counting a total of 300 sperm under a phase-contrast microscope (× 1000 magnification; oil immersion).

2.7. Lipid peroxidation

Malondialdehyde (MDA) concentrations in semen samples were measured using the thiobarbituric-acid reaction [40]. Briefly, 1 ml of the diluted semen sample (250×10^6 sperm/ml) was mixed with 1 ml of cold 20% (w/v) tricholoro acetic acid to precipitate protein. The precipitate was pelleted by centrifuging (960 g for 15 min), and 1 ml of the supernatant was incubated with 1 ml of 0.67% (w/v) thiobarbituric acid in a boiling water bath at 100 °C for 10 min. After cooling, the absorbance was determined by a spectrophotometer (UV-1200, Shima-dzu, Japan) at 532 nm. All MDA concentrations were expressed as nmol/ml.

2.8. Evaluation of phosphatidyl serine externalization

For determination of externalization of phosphatidyl serine as an earliest indicator of apoptotic like changes in the semen sample, the sperm samples were washed in calcium buffer and the concentration was readjusted to 1×10^6 sperm/ml, followed by the addition of 10 ml Annexin V–FITC (0.01 mg/ml) to 100 ml of the sperm suspension [39]. This was then incubated for 20 min on ice. Next, 10 ml of propidium iodide (PI; 1 mg/ml) was added to the sperm suspension and incubated for at least 10 min on ice. Then, suspension was evaluated by flow cytometer. The sperm samples were classified into four groups: (1) viable nonapoptotic cells negative for both Annexin V and PI (A^-/PI^-); (2) early apoptotic cells positive for Annexin V but negative for PI (A^+/PI^-); (3) late apoptotic cells positive for both Annexin V and PI (A^+/P^+); and (4) necrotic cells negative for Annexin V but positive for PI (A^-/P^+). The late apoptotic and necrotic cells were categorized as dead cells.

2.9. Mitochondrial activity

The percentage of sperm with active mitochondria was determined by Rhodamine 123 (R123; Invitrogen TM, Eugene, OR, USA) and PI as describe by Najafi et al. (2013). TM, Eugene, OR, USA) and

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PI were used to evaluate mitochondrial activity. Ten microliters of Rhodamin-123 solution (0.01 mg/ml) was added to 300 μl of diluted semen samples by Tris buffer and incubated for 20 min in the dark room. Then, sperm suspensions were centrifuged at 500 × g (3 min) and resuspended in 500 μl Tris buffer. Then, 10 μl of PI (1 mg/ml) was added to sperm suspensions. For each sample, 10000 events were recorded. Sperms with active mitochondria were identified by positive signal for Rh123 and negative signal for PI.

2.10. Flow cytometry procedure

Flow cytometry analyses of apoptosis and mitochondrial activity were performed using the FACSCalibur (Becton Dickinson, San Khosoz, CA, USA) flow cytometer equipped with standard optics. A minimum of 10,000 sperms were examined for each assay at a flow rate of 100 cells/s. The sperm population was gated using 90° and forward-angle light scatter to exclude debris and aggregates. The excitation wavelength was 488 nm supplied by an argon laser at 250 mW. Green fluorescence (FL1) was measured using a 530/30 nm bandpass filter and red fluorescence (PI, FL3) was measured by a 585/42 nm filter and Rh123 was measured using a 527/25 nm filter (FL3). The analysis of flow cytometry data was performed using FlowJo software (Treestar, Inc., San Carlos, CA).

2.11. Statistical analysis

All data were analyzed using general linear model procedure using SAS 9.1 [36]. Six replicates were used for evaluation. Statistical differences among various group means were determined by the Tukey's test and the values of $P < 0.05$ were considered to be statistically significant. Results are shown as Mean ± SEM.

3. Results

3.1. Motion and velocity

Table 1 shows the effects of different concentrations of LC on the motility and velocity parameters of rooster sperm after freeze-thawing. LC at concentrations of 1 and 2 mM resulted in significantly ($P < 0.05$) higher total motility (68.2 ± 1.7 and 69.1 ± 1.7, respectively) and progressive motility (28.4 ± 1.6, 29.8 ± 1.6, respectively) compared to control group. There was no significant difference between control and L0.5 for total motility (52.7 ± 1.7 vs. 55.7 ± 1.7) and progressive motility (19.4 ± 1.6 vs. 21.2 ± 1.6). Furthermore, total (35.8 ± 1.7) and progressive motility (9.6 ± 1.2) were significantly ($P < 0.05$) lower in L8 compared to control and other treatments. For the straight line velocity (VSL), there was no significant difference between control, L0.5, L1 and L2 (15.7 ± 2.1, 16.5 ± 2.1, 18.2 ± 2.1 and 17.3 ± 2.1, respectively), while the lowest VSL was produced in the L8 (5.0 ± 2.1). For VAP, VCL and LIN no significant difference was observed between groups.

3.2. Membrane integrity, mitochondria activity and normal morphology

Effects of different concentrations of LC on sperm membrane functionality, mitochondria activity and abnormal morphology are shown in Table 2. Higher ($P < 0.05$) significant of membrane functionality after freeze-thawing was observed in groups containing 1 and 2 mM LC (76.2 ± 1.9 and 75.9 ± 1.9, respectively) compared to control (63.4 ± 1.9), L0.5 (65.1 ± 1.9), L4 (59.6 ± 1.9) and L8 (57.8 ± 2.1). No significant difference in membrane functionality was observed between control, L0.5, L4 and L8.

Supplementation of freezing media with various concentrations of LC had no effect on mitochondrial activity (R123 positive, MAP) and morphology of rooster frozen-thawed sperm ($P > 0.05$).

3.3. Lipid peroxidation

Results of MDA production in freeze-thawed sperm are shown in Table 2. L1 and L2 decreased LPO (2.53 ± 0.08 and 2.49 ± 0.08) compared to control and L0.5 (2.80 ± 0.08 and 2.82 ± 0.08). Moreover, L4 and L8 increased LPO compared to other treatments (3.05 ± 0.08 and 3.10 ± 0.08).

3.4. Phosphatidyl serine externalization

The results of Annexin V/PI analysis after sperm cryopreservation under different concentrations of LC are shown in Fig. 1. The viability rates were significantly ($P < 0.05$) higher in L1 and L2 (58.2 ± 1.1, 59.1 ± 1.1, respectively) compared to control. Furthermore, L1 and L2 produced the lowest significant percentage of apoptotic-like changes compared to control group. The percentage of dead sperm after freeze-thawing was not affected by groups.

4. Discussion

In this study, we have shown that the presence of LC concentrations (1–2 mM) in cryopreservation media increased rooster sperm motility, membrane functionality, viability and lipid peroxidation after freeze-thawing. Higher motility and membrane functionality of sperm that are treated with LC prior cryopreservation may be attributed to the metabolism role of LC [5]. LC facilitates the transport of activated fatty acids across the inner membrane of mitochondria, allowing their β-oxidation to produce ATP [41], thereby supplying energy for movement of sperm. Moreover, accumulation of ROS in sperm leads to destruction of membrane as well as depletion in the ATP supply, which can negatively affect sperm quality [13]. Therefore, these characteristics of LC could be responsible for improving the motility and membrane functionality of rooster sperm during cryopreservation.

It has been also reported that the pyruvate system is more efficient when LC is accessible for sperm [43]. Therefore, another reason of higher motility and membrane functionality of sperm in this study may be related to higher efficiency of the pyruvate system in rooster sperm.

In an earlier study, the characteristics of frozen-thawed epididymal cat sperm was found to be better when LC was added to cat sperm extender [21]. Similarly, LC supplementation of human sperm before cryopreservation has been shown to have increased sperm motility, which is in accordance with the results of our study [49]. However, Duru et al. reported that treatment of human semen with LC did not have any significant effect on sperm motility and membrane functionality [14]. This discrepancy may be related to various factors such as species, components of extenders and procedure of freezing. LC can also stabilize the plasma membrane of the sperm, possibly via interaction with membrane phospholipid, which modulates fluidity of plasma membrane. This phenomenon can allow the sperm to resist damages during cryopreservation. This improvement in integrity and functionality of sperm could result in better characteristics of sperm such as motility.

We applied Annexin-V/PI assessment to accurately detect sperm with phosphatidyl serine (PS) translocation as a sign of apoptotic like changes. Phosphatidyl serine externalizations have been found to be enhanced during cryopreservation [2]. Our hypothesis was that LC may reduce apoptotic markers such as PS due to stabilization effect of LC on the sperm plasma membrane. Our data in this part is in agreement with earlier reports that LC reduced the apoptotic spermatogonia stem cells in rats fed on a diet

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Table 1
Effects of LC (mM) on the motion parameters of rooster semen in the Beltsville extender.

Variable	Groups					
	0	0.5	1	2	4	8
Total Motility	52.7 ± 1.7[b]	55.7 ± 1.7[b]	68.2 ± 1.7[a]	69.1 ± 1.7[a]	43.1 ± 1.7[c]	35.8 ± 1.7[d]
Progressive (%)	19.4 ± 1.6[b]	21.2 ± 1.6[b]	28.4 ± 1.6[a]	29.8 ± 1.6[a]	16.2 ± 1.7[b]	9.6 ± 1.2[c]
VAP (μm/s)	27 ± 2.8	29.1 ± 2.8	28.7 ± 2.8	26.5 ± 2.8	24.2 ± 2.8	25.3 ± 2.8
VSL (μm/s)	15.7 ± 2.1[a]	16.5 ± 2.1[a]	18.2 ± 2.1[a]	17.3 ± 2.1[a]	11.4 ± 2.1[b]	5 ± 2.1[c]
VCL (μm/s)	22.3 ± 3.6	21.5 ± 3.6	23.2 ± 3.6	20.5 ± 3.6	19.8 ± 3.6	20.7 ± 3.6
LIN (%)	29.3 ± 1.5	27.1 ± 1.5	30.7 ± 1.5	26.4 ± 1.5	29.8 ± 1.5	27.4 ± 1.5

Different letters within the same row show significant differences among the groups ($p \leq 0.05$).

supplemented with LC [17]. In a similar study in human, higher vitality of sperm was obtained after cryopreservation in the presence of LC [4].

An analysis of lipid peroxidation of rooster sperm in freezing media containing different concentrations of LC shows that MDA levels were lower in groups containing 1–2 mM LC. We also observed an antioxidant effect of LC during cryopreservation of rooster sperm. This characteristic of LC is related to its ability to chelate free ferrous ions, inhibit generated superoxide ions and detoxify accumulated ROS [4]. This result is in agreement with several earlier studies that have reported the effects of extender additives on frozen-thawed sperm lipid peroxidation [9,51]. It seems that LC protects sperm effectively against lipid peroxidation, which can be related to the metabolism and biochemical roles of LC that are crucial in sperm plasma membranes. However, this result is in contrast to those by Atessahin et al. who postulated that lipid peroxidation in sperm is not affected by the ingredients of cryopreservation media [3]. This discrepancy may be related to the differences in cryopreservation procedure and applied protocols, which can effect on the lipid peroxidation.

An interesting observation related to LPO production is the suddenly increment of MDA at higher concentrations of LC (LC4 and LC8). Similar data were also observed where the higher concentrations of LC were shown to be toxic for cat epididymis sperm [21]. It seems that high concentrations of LC can increase the fluidity of plasma membrane above the desired point, making sperm more

Table 2
Percentage of membrane functionality (MF), mitochondria activity (MA), normal morphology (NM) and lipid peroxidation (LPO) of sperm after freeze-thawing in the extender containing different concentrations of LC (mM).

Variable	L-Carnitine Concentrations (mM)					
	0	0.5	1	2	4	8
MF (%)	63.4 ± 1.9[b]	65.1 ± 1.9[b]	76.2 ± 1.9[a]	75.9 ± 1.9[a]	59.6 ± 1.9[b]	57.8 ± 2.1[b]
MA (%)	64 ± 3.4	59.4 ± 3.5	66.7 ± 3.5	60.7 ± 3.5	65.3 ± 3.5	59 ± 3.5
NM (%)	83.24 ± 2.5	89.19 ± 2.5	90.14 ± 2.5	90.47 ± 2.5	88.12 ± 2.5	85.3 ± 2.5
LPO (nmol/ml)	2.80 ± 0.08[b]	2.82 ± 0.08[b]	2.53 ± 0.08[a]	2.49 ± 0.08[a]	3.05 ± 0.08[c]	3.10 ± 0.08[c]

Different letters within the same rows show significant differences among the groups ($p \leq 0.05$).

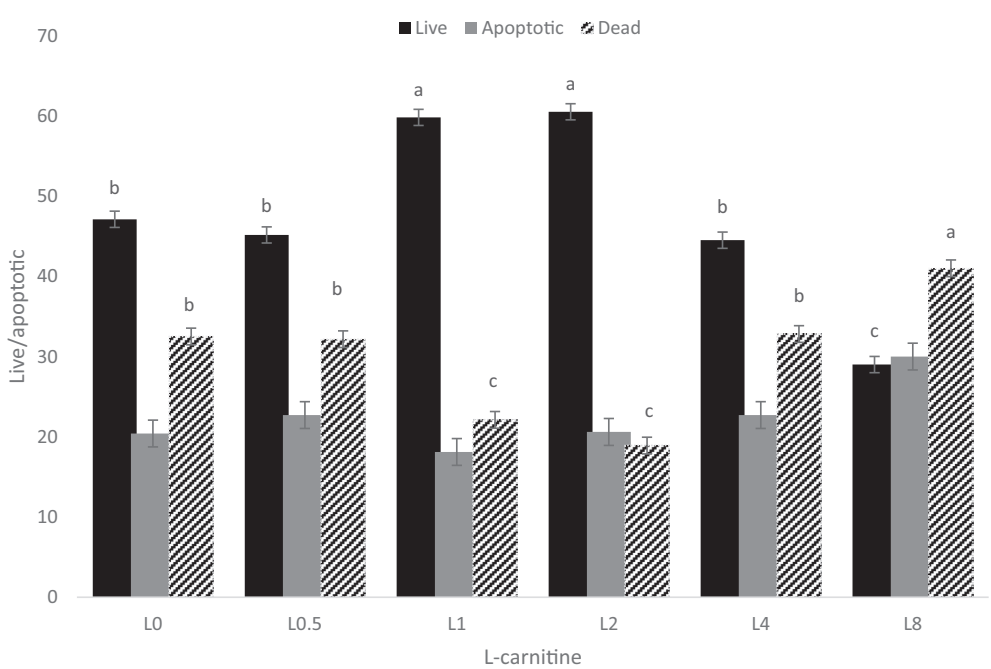

Fig. 1. Viability and apoptosis status of rooster sperm after freeze-thawing using the extender that contained different concentrations of LC.

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susceptible to lipid peroxidation. However, further studies are required to identify the exact molecular mechanism of the harmful effects of high amounts of LC.

In our trial, LC did not have any effect on the morphology of the sperm, when compared to that of the control group. It seems that secondary morphology of sperm are not affected by the processing technique because primary sperm abnormalities occurs during spermatogenesis [12]. Our findings are in agreement with Bucak et al. (2010) who reported that in vitro supplementation of goat semen extender with LC did not have any effect on the normal percentage of sperm cells after freezing [7]. However, beneficial effects of dietary LC on the morphology of stallion sperm have been reported [42].

It has been thought that mitochondria supplies the energy needed for sperm motility [31]. Assessment of frozen-thawed rooster sperm with R123 fluorescent dye indicated that the percentage of mitochondrial activity of sperm was not affected by LC. While we expected a logical relationship between sperm motility and mitochondrial activity, we observed a relatively independent response in mitochondrial activity from motility parameter as reported by other researchers [23]. This finding is in agreement with earlier suggestions that motion characteristics of sperm are independent of mitochondrial activity [22,48].

5. Conclusion

The results of this study indicated that higher motility, membrane functionality and viability of rooster sperm after freeze-thaw were obtained by supplementation of extender with 1 and 2 mM LC. However, fertilizing ability of sperm is important, and further studies must be aimed at evaluating the effects of LC on fertility parameters.

Conflict of interest

None of the authors have any conflict of interest to declare.

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