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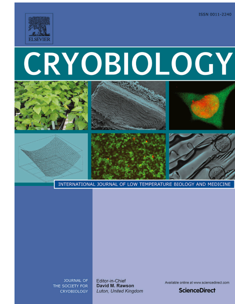

L-carnitine is a survival factor for chilled storage of rooster semen for a long time

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1 **Highlighted Revised**

L-carnitine is a survival factor for chilled storage of rooster semen for a long time

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22 # L-carnitine is a survival factor for chilled storage of rooster semen for a long
23 time

24 **Abstract**

25 Rooster sperm is sensitive to cooling, which restricts procedures to store sperms for extended
26 periods of time for artificial insemination of commercial flocks. This study was conducted to
27 evaluate the suitability of adding L-carnitine (LC) to chilled-storage of rooster sperm and its
28 effects on sperm quality parameters and its fertility potential during storage at 5°C. Pooled semen
29 from roosters were divided into six equal aliquots and diluted with media supplemented with
30 different concentrations of LC (0, 0.5, 1, 2, 4 and 8 mM LC). Diluted semen samples were
31 cooled to 5°C and stored over 48 h. Motility, viability, membrane functionality, lipid
32 peroxidation and mitochondria activity of the sperm were assessed at 0, 24 and 48 h of storage.
33 Moreover, fertility potential of chilled stored sperm was considered at 24 h of storage. While
34 sperm quality was not affected by LC at the beginning of storage (0 h), supplementation of
35 extender with 1 and 2 mM of LC significantly improved the percentage of sperm motility,
36 viability, membrane integrity and mitochondria activity at 24 h and 48 h compared to other
37 groups. Lipid peroxidation was significantly reduced in sperm samples diluted with 1 and 2 mM
38 LC at 24 h (2.15 ± 0.52 nmol/ml and 2.21 ± 0.52 nmol/ml) and 48 h (3.42± 0.49 nmol/ml and
39 3.38 ± 0.49 nmol/ml) compared to other groups. Furthermore, fertility rates during artificial
40 insemination using sperms cooled for 24 hours in the presence of 1 and 2 mM LC were
41 significantly higher (78%) than in the control group (64%). These findings suggest that optimum
42 doses of LC could protect rooster sperm against cool storage-induced functional and structural
43 damages.

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44 **Keywords:** Rooster; Cooling storage; L-carnitine; Sperm; Fertility.

45 **Introduction**

46 Liquid storage of semen with preservative extenders is used to reduce sperm metabolism and
47 preserve sperm viability over a long time [43]. Although several studies have attempted to
48 optimize procedures for cold-storage of rooster sperms for more than 24 hours [17,27,43],
49 fertility rates have been unsatisfactory when hens are inseminated with semen stored for more
50 than 6 h [35].

51 The poor fertility rate associated with stored sperm has been attributed to unique physiological
52 features of rooster sperm, which may be damaged during cooling preservation [36]. Rooster
53 sperm may also be exposed to chilling-injuries that lead to membrane damage and consequent
54 reduction of fertility potential [38]. Therefore, it is crucial to improve and optimize procedures to
55 protect rooster sperm during liquid storage. The most widely used diluent for preservation of
56 rooster sperm is Beltsville Poultry Semen Extender (BPSE), which is composed of dipotassium
57 phosphate, sodium glutamate, fructose, and sodium acetate, in addition to other buffers and salts
58 [35]. However, it is necessary to add an additive to this extender to protect rooster sperm against
59 chilling stress, a process that can resulted in production of reactive oxygen species which leading
60 to distraction of sperm structure [25,26].

61 In this study, we have attempted to reinforce BPSE using L-carnitine (LC) because of its
62 metabolic and antioxidant roles. LC is a water-soluble vitamin-like amino acid, which plays
63 crucial roles in generation of sperm metabolic energy by facilitating the transport of fatty acids
64 into the mitochondria [14]. LC also has antioxidant characteristics associated with the
65 stabilization of mitochondrial membrane and protects the DNA structure against ROS [29]. This
66 property is related to the ability of LC to reduce the availability of lipids for peroxidation by

67 transportation of fatty acids into the mitochondria for β-oxidation [11,24]. Dietary LC has been
68 reported to enhance the activity of antioxidant enzymes such as superoxide dismutase and
69 glutathione peroxidase in sperm [27]; these enzymes play important roles in scavenging of ROS
70 in chilled sperm. Earlier studies have shown the beneficial effects of LC on sperm motility in
71 human [3], boar [46], quail [31], stallion [12], bull [32], rainbow trout [16] and chicken [27].
72 There have been, to the best of our knowledge, no studies have investigated the effects of LC-
73 supplemented BPSE on motility, viability, membrane functionality, lipid peroxidation and
74 fertility response of sperms during chilled storage. In this work, we have investigated
75 mitochondria active potential (MAP) of chilled sperms in the presence/absence of LC, through
76 flow cytometric procedures, as described in the following sections.

77 **Materials and methods**

78 *Chemicals*

79 All chemicals used in this study were purchased from Sigma (St. Louis, MO, USA) and Merck
80 (Darmstadt, Germany) unless those mentioned. Approval for the present study was given by the
81 Research Ethics Committees of Royan Institute.

82 *Farm management and semen collection*

83 This experiment was performed on Ross broiler breeders represented by 12 adult males (32
84 weeks), kept individually in cages (70×60×75 cm) at 18-22 °C, under 15 L: 09 D photoperiod.
85 Animals were fed on a diet containing 10% crude protein, 3170 kcal ME/kg, 0.9 % calcium and
86 0.45% available phosphate. Semen was collected from 12 roosters twice a week during four
87 weeks using abdominal massage according to the method of Burrows and Quinn [5] and then
88 transferred to a water bath (37°C) for primary evaluation. The criteria for normal quality of

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89 sperm were as follows: volume: 0.2-0.6 ml; sperm concentration of $\geq 3\times 10^9$ spermatozoa/ml;
90 motility ≥80% and abnormal morphology ≤10%. Then, to eliminate individual differences,
91 semen samples were pooled and divided into six aliquots according to the experimental design.

92 *Extender preparation*

93 The components of control extender (BPSE) were dipotassium phosphate (7.59 g/l), sodium
94 glutamate (8.67 g/l), fructose (5 g/l), sodium acetate (3.2 g/l), TES [n-tris (hydroxymethyl)
95 methyl 1-2 amino ethane sulfonic acid] (3.2 g/l), potassium citrate (0.64 g/l), monopotassium
96 phosphate (0.7 g/l), magnesium chloride (0.34 g/l) [35]. pH and osmolarity were set at 7.1 and
97 310 mOsm/kg, respectively. Experimental groups in this study were as follows: BPSE without
98 LC (control), BPSE with 0.5 mM (LC0.5), 1 mM (LC1), 2 mM (LC2), 4 mM (LC4) and 8 mM
99 (LC8) LC. Sperm samples were diluted with media (37°C) according to experimental groups, at a
100 final concentration of 400×10^6 sperm/ml and then placed in a rack and cooled from 37 to 5°C for
101 30 minutes, in a cold cabinet, and maintained at 5 ^{o}C. Motility, viability, membrane
102 functionality, mitochondria activity and lipid peroxidation were evaluated at 0 (at the beginning
103 of the cooling process), 24 and 48 h of storage.

104 *Evaluation of sperm parameters during storage*

105 *Motility, viability and membrane functionality*

106 Sperm class analysis software (Version 5.1; Microptic, Barcelona, Spain) was used to analyze
107 sperm motility. For this purpose, 5 µl of diluted semen was placed into a prewarmed chamber
108 slide (38 ^{o}C, Leja 4; 20 mm height; Leja Products, Luzernestraat B.V., Holland [19]. At least six
109 fields that contained a minimum of 400 sperm were evaluated for each sample at a 5 sec average
110 time to read each sample. The following values were recorded: motility (%), progressive motility

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111 (%), average path velocity (VAP, µm/sec), straight linear velocity (VSL, µm/sec), curvilinear
112 velocity (VCL, µm/sec), and amplitude of lateral head displacement (ALH, µm).

113 Viability was assessed using eosin–nigrosine staining, by counting 200 spermatozoa for
114 unstained heads of spermatozoa (live) and/or stained/partial stained heads of spermatozoa (dead)
115 under phase-contrast microscope at 400 x [23].

116 For evaluation of membrane functionality, Hypo Osmotic Swelling Test (HOST) was performed
117 according to the method described by Revell and Mrode (1994) with a slight modification [30].
118 This assay was carried out by mixing 5 µl of semen with a 50 µl hypo osmotic solution (100
119 mOsm/l, 57.6 mM fructose and 19.2 mM sodium citrate). After 30 min incubation, the sperm
120 were checked under a phase-contrast microscope (CKX41, Olympus, Tokyo, Japan) and 300
121 sperm with swollen and non-swollen tails were recorded as sperm with integrated and non-
122 integrated membrane, respectively.

123 *Lipid peroxidation*

124 Malondialdehyde (MDA) concentrations in diluted semen were measured as an index of lipid
125 peroxidation using the thiobarbituric-acid reaction [10]. Briefly, 1 ml of the diluted semen (400
126 $\times 10^6$ sperm/ml) was mixed with 1 ml of cold 20% (w/v) trichlo acetic acid to precipitate protein.
127 The precipitate was pelleted by centrifuging (960g for 15 min), and 1 ml of the supernatant was
128 incubated with 1 ml of 0.67% (w/v) thiobarbituric acid in a boiling water bath at 100 ^oC for 10
129 min. After cooling, the absorbance was determined by a spectrophotometer (UV-1200, Shima-
130 dzu, Japan) at 532 nm. All MDA concentrations were expressed as nmol/ml.

131 *Mitochondria activity with flow cytometry analysis*

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132 Mitochondria potential of sperm during liquid storage was determined by Rhodamine 123
133 (R123; Invitrogen TM, Eugene, OR, USA) and propidium iodide (PI) as previously described
134 [21]. Ten microliters of Rhodamine-123 solution (0.01 mg/ml) was added to 300 µl of diluted
135 semen samples and incubated for 20 min in the dark room. Then, the sperm suspension was
136 centrifuged at 500 x g (3 min), and again resuspended in 500 µl Tris buffer. Then, 10 µl of PI (1
137 mg/ml) was added to sperm suspension. Flow cytometry analyses of mitochondrial activity
138 were performed using the FACSCalibur (Becton Dickinson, San Khosoz, CA, USA) flow
139 cytometer equipped with standard optics. A minimum of 10,000 sperms were examined for each
140 assay at a flow rate of 100 cells/s. The sperm population was gated using 90° and forward-angle
141 light scatter to exclude debris and aggregates. The excitation wavelength was 488 nm supplied
142 by an argon laser at 250 mW. Rh123 (FL1) and red fluorescence (PI, FL3) were measured using
143 527/25 and 585/42 nm filters, respectively. The analysis of flow cytometry data was performed
144 using FlowJo software (Treestar, Inc., San Carlos, CA).

145 *In vivo fertility evaluation*

146 Artificial insemination was conducted according the procedure of Long et al. [18] with a little
147 modification. Ross broiler breeder hens were divided into three groups (20 hens per group),
148 which were housed in individual cages (70 × 70 × 85 cm) and inseminated with sperm stored in
149 three experimental treatments that were selected according to the results of in-vitro sperm
150 parameters. LC concentrations of 1 and 2 mM (1 and 2) along with control group were applied to
151 test the fertility potential of rooster stored sperm at 24 h of incubation. Since the sperms were of
152 same quality at the start of incubation, this time was deleted for fertility trials. Moreover, 48 h of
153 incubation was also eliminated for fertility test because the quality of sperm was not suitable for
154 this purpose. We used 0.25 ml of semen (100×10^6 sperm) for insemination, twice per week for

155 one month. The eggs were collected up to five days after the last artificial insemination. For each
156 group, totally 400 eggs in 4 weekly sets (100 eggs in each set) were selected for incubation. On
157 day 7 of incubation, the fertility rate was measured by candling the eggs. Hatching rate was
158 calculated after 21 days of incubation based on number of eggs.

159 *Statistical analysis*

160 Six replicates of semen were used for in vitro evaluation of sperm parameters. All data were
161 checked for normal distribution by Shapiro–Wilk test and analyzed using Proc GLM of SAS 9.1
162 [34] (SAS Institute, version 9.1, 2002, Cary, NC, USA). Statistical differences among various
163 group means were determined by Tukey's test and the values of $P<0.05$ were considered to be
164 statistically significant. Results were shown as Mean±SE. Fertility and hatching rate were
165 analyzed via GENMOD procedure using Chi-Square.

166 **Results**

167 *Motility*

168 Table 1 shows the effects of different concentrations of LC on total motility of rooster sperm
169 during chilled storage at different times.

170 There was no significant difference between experimental groups at beginning of cooling (0h).
171 At 24 h, LC1 and LC2 showed significantly ($P<0.05$) higher total motility (60.3 ± 1.7% and 63.5
172 ± 1.7%, respectively) compared to control (51.7 ± 1.7%), LC0.5 (49.1 ± 1.7%), LC4 (46.5 ±
173 1.7%) and LC8 (37.1 ± 1.7%). The lowest significant total motility at 24 h was observed in LC8
174 (37.1 ± 1.7%). The higher significant ($P<0.05$) total motility at 48 h was obtained in LC2 (28.2 ±
175 2.3%) compared to other groups. At this time, total motility values in control (19.7 ± 2.3%),

176 LC0.5 (18.4 ± 2.3%) and LC1 (20.7 ± 2.3%) were significantly (P<0.05) higher than LC4 (11.2
177 ± 2.3%) and LC8 (3.7 ± 2.3%).

178 *Viability*

179 Data related to the effects of LC on sperm viability during cooling storage are presented in table
180 2. No significant difference was found among LC groups at the beginning time of cooling (0 h).
181 However, the higher significant (P<0.05) viability at 24 h storage was observed in LC1 and LC2
182 (65.3 ± 2.4% and 69.5 ± 2.4%, respectively) compared to control (57.8 ± 2.4%), LC0.5 (54.1±
183 2.4%), LC4 (50.5 ± 2.4%) and LC8 (40.1 ± 2.4%). Semen diluted in LC8 had the lowest viability
184 rate (40.1 ± 2.4%), while the differences between LC0, LC0.5 and LC4 were not significant.

185 Viability percentage of diluted semen at 48 h was significantly higher (P<0.05) in LC2 (33.2 ±
186 2.6%) than other groups. Moreover, viability percentages in control (25.8 ± 2.6%), LC0.5 (22.4 ±
187 2.6%) and LC1 (26.7 ± 2.6%) were significantly (P<0.05) higher than for LC4 (14.2 ± 2.6%) and
188 LC8 (5.7 ± 2.6%).

189 *Membrane functionality*

190 Table 3 shows the effects of LC on sperm membrane functionality during cooling storage.
191 Differences in membrane functionality of sperm among the different groups were not significant
192 at the beginning time of cooling (0 h). After 24 h, higher (P<0.05) significant membrane
193 functionalities were observed in LC1 and LC2 (70.3 ± 2.7% and 68.5 ± 2.7%, respectively)
194 compared to control (59.8 ± 2.7%), LC0.5 (60.6 ± 2.7%), LC4 (56.5 ± 2.7%) and L8 (45.1 ±
195 2.7%). LC1 and LC2 showed also significantly (P<0.05) higher membrane functionalities (33.7 ±
196 1.8% and 35.6 ± 1.8%, respectively) at 48 h compared to control (29.8 ± 1.8%), LC0.5 (28.4 ±

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197 1.8%), LC4 (17.2 ± 1.8%) and LC8 (11.5 ± 1.8%). The lowest membrane functionalities of
198 sperm at 24 and 48 hours were observed in LC 8.

199 *Lipid peroxidation*

200 Lipid peroxidation (table 4) was not significantly affected by LC at the beginning of cooling (0
201 h), but after 24 hours, lower ($P<0.05$) MDA values were observed in LC1 (2.15 ± 0.52 nmol/ml)
202 and LC2 (2.21 ± 0.52 nmol/ml) compared to control (3.81 ± 0.52 nmol/ml), LC0.5 (3.67 ± 0.52
203 nmol/ml), LC4 (3.76 ± 0.52 nmol/ml) and LC8 (3.84 ± 0.52 nmol/ml). Furthermore, after 48 h of
204 storage in 5 ^{o}C, the amounts of lipid peroxidation in LC1 (3.42 ± 0.49 nmol/ml) and LC2 (3.38 ±
205 0.49 nmol/ml) were lower ($P<0.05$) than control (4.76 ± 0.49 nmol/ml), LC0.5 (4.78 ± 0.49
206 nmol/ml), LC4 (4.85 ± 0.49 nmol/ml) and LC8 (4.91 ± 0.49 nmol/ml).

207 *Mitochondria active potential*

208 Results of sperm mitochondria active potential (MAP) are presented in table 5. Various
209 concentrations of LC did not have any effect on MAP at 0 h. Twenty-four hours after dilution,
210 MAP values were significantly ($P<0.05$) higher in LC0.5 (66.1 ± 1.5%), LC1 (66.3 ± 1.5%) and
211 LC2 (69.5 ± 1.5%) than control (60.1 ± 1.5%), LC4 (55.7 ± 1.5%) and LC8 (34.1 ± 1.5%).
212 Moreover, MAP was significantly ($P<0.05$) higher in LC0.5 (18.4 ± 1.7%), L1 (18.7 ± 1.7%) and
213 LC2 (19.2 ± 1.7%) than control (13.8 ± 1.7%), LC4 (8.2 ± 1.7%) and LC8 (2.3 ± 1.7%) 48 hours
214 after dilution.

215 *Fertility rate*

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216 For fertility potential (table 6), higher significant rates of fertility and hatching were obtained in
217 LC1 (78 and 69%) and LC2 (80 and 73%) compared to control group (64 and 56%). The
218 difference between LC1 and LC2 was not significant.

219 Discussion

220 Chilled storage of semen for extended periods of time can cause several time-dependent
221 structural and biochemical damages to sperm in avian [27] and mammalian species [12, 32].
222 Therefore, the semen must be diluted with an appropriate medium enriched with effective
223 protective supplements [39,40]. BPSE has been widely shown in earlier studies, to be effective
224 for preservation of chicken sperm under different states of liquid storage [38]. In the present
225 study, we have investigated the potential beneficial effects of LC added to BPSE, for rooster
226 sperms stored at 5°C for over 48 h. We observed a time-dependent reduction in motility,
227 viability, membrane functionality and mitochondria activity of rooster sperm diluted in all
228 experimental groups. However, this reduction in groups containing 1-2 mM LC was less than
229 other groups. Supplementation of BPSE with 1-2 mM LC in our study produced higher motility,
230 viability and membrane functionality as well as lower lipid peroxidation compared to control and
231 other concentrations of LC at 24 and 48 h of incubation. Furthermore, we obtained comparable
232 fertility rates with sperm supplemented with 1 and 2 mM LC at 24 h of incubation.

233 Several studies have reported that oral or dietary consumption of LC increases semen quality in
234 human [45] and chicken [27] sperm. Moreover, supplementation of semen extenders with LC
235 have been tested for stallion [12], bull [32] and rabbit [33] sperm while there are notably few
236 studies that have investigated the effect of in vitro supplementation of LC on breeder rooster
237 sperm as well as its fertility potential [43]. Higher motility, viability and membrane functionality

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238 of sperm treated with 1-2mM LC may be attributed to the role of LC in metabolism [4].
239 Facilitation in fatty acids transport across the inner membrane of mitochondria via LC leads to
240 improve the production of ATP by β-oxidation [41], thus providing better supply of energy for
241 sperm motility. On the other hand, ROS accumulated during storage of sperm [8] are probably
242 scavenged by an optimum dose of LC, also resulting in lower damages to sperm during chilled
243 storage. LC is found in higher concentrations in the seminal plasma compared to blood plasma
244 [15], suggesting the crucial role of LC in the pyruvate cycle in production of energy [42].

245 Antioxidant characteristics of LC arise from the scavenging of free radicals, destruction of
246 hydrogen peroxide and metal chelation as well as inhibition of xanthine oxidase activity by LC
247 [13]. Of these properties, reduction of lipid peroxidation is reported in literature and is normally
248 considered in research and clinical attempts [7,8]. Our results related to lipid peroxidation
249 verified the data obtained for sperm motility, viability and membrane functionality because
250 MDA concentrations were lower in LC 1-2 mM, which in agreement with previous reports of
251 beneficial effects of LC on sperm [1,3] and other cell types [9]. However, this result is
252 contradictory to observations of Atessahin et al. [2], who reported that lipid peroxidation in goat
253 sperm is not affected by media ingredients. This discrepancy may be related to the sperm type
254 and applied protocol, which can effect on the lipid peroxidation results.

255 Our results also showed a logical relationship between sperm motility and mitochondrial activity.
256 Sperm motility has been found to be relatively dependent on mitochondria activity, as suggested
257 by other researchers [20,22]. This phenomenon may be due to osmolyt role of LC in the
258 extender. In fact, supplementation of diluent with LC leads to partial removal of Na from diluent
259 to maintain isotonicity [37]. Because Na increases the depletion of ATP via activation of Na-
260 ATPase pumps [37], it is suggested that the beneficial effects of LC may be due to the removal

261 of Na [12]. It has also been suggested that reduction of Na in solution reduces the energy
262 demands of the cell sperm resulting in slower rate of ATP depletion, effectively improving and
263 maintaining sperm viability and mitochondria activity for extended periods of time [12].

264 The fertility and hatching rates obtained using chill-preserved sperms in extender that contained
265 1 and 2mM LC were 75% and 66% respectively. Such rates are comparable to fertility studies
266 that use fresh semen for artificial insemination [28,36]. There was a significant improvement in
267 fertility rate when 1 and 2 mM LC were compared to control extender without LC. This
268 improvement is related to higher quality of sperm treated with LC, which can directly affect the
269 results of fertility. Our fertility results with addition of 1 and 2 mM LC to BPSE were similar to
270 the results reported in an earlier fertility trial experiment [36]. It must be remembered however,
271 that numerous factors such as nature of the hen, the technique used, number of spermatozoa,
272 environmental factors, depth of semen deposition and frequency of insemination can affect
273 fertility rate after artificial insemination [6,17,44].

274 **Conclusion**

275 Supplementation of rooster sperm diluent (BPSE) with LC preserves the quality of cold-stored
276 semen by supporting mitochondrial active potential while reducing the amount of lipid
277 peroxidation. Furthermore, addition of optimum doses of LC to BPSE preserved the fertility
278 potential of sperm during cooling preservation. This study also suggests a practical way by
279 which rooster semen can be transported to far-away farms for insemination of commercial
280 flocks, without significant losses in viability, motility and fertilization efficacy.

281 **Conflict of interest**

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

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420 Table 1. Effects of L-Carnitine (mM) on rooster sperm motility (M±SEM) during 0, 24 and 48 h
421 of storage at 5°C. (M±SEM)

LC concentrations	0	24	48
0	89.5 ± 2.9	51.7 ± 1.7[b]	19.7 ± 2.3[b]
0.5	92.7 ± 2.9	49.1± 1.7 [b]	18.4 ± 2.3 [b]
1	90.8 ± 2.9	60.3 ± 1.7[a]	20.7 ± 2.3[b]
2	86.4 ± 2.9	63.5 ± 1.7 [a]	28.2 ± 2.3[a]
4	92.3 ± 2.9	46.5 ± 1.7[b]	11.2 ± 2.3[c]
8	88.6 ± 2.9	37.1 ± 1.7[c]	3.7 ± 2.3[d]

422 Different letters within the same column show significant differences among the groups (P≤0.05).

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425 Table 2. Effects of L-Carnitine (mM) on rooster sperm viability (M±SEM) during 0, 24 and 48 h
426 of storage at 5°C.

LC concentrations	0	24	48
0	91.5 ± 1.9	57.8 ± 2.4[b]	25.8 ± 2.6[b]
0.5	90.7 ± 1.9	54.1± 2.4 [b]	22.4 ± 2.6 [b]
1	92.8 ± 1.9	65.3 ± 2.4[a]	26.7 ± 2.6[b]
2	90.4 ± 1.9	69.5 ± 2.4 [a]	33.2 ± 2.6[a]

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4	92.3 ± 1.9	50.5 ± 2.4^{b}	14.2 ± 2.6^{c}
8	90.0 ± 1.9	40.1 ± 2.4^{c}	5.7 ± 2.6^{d}

427 Different letters within the same column show significant differences among the groups (P≤0.05).

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431 Table 3. Effects of L-Carnitine (mM) on rooster sperm membrane functionality (M±SEM)
432 during 0, 24 and 48 h of storage at 5°C.

LC concentrations	0	24	48
0	90.3 ± 1.4	59.8 ± 2.7^{b}	29.8 ± 1.8^{b}
0.5	89.7 ± 1.4	60.6± 2.7^{b}	28.4 ± 1.8^{b}
1	91.8 ± 1.4	70.3 ± 2.7^{a}	33.7 ± 1.8^{a}
2	90.6 ± 1.4	68.5 ± 2.7^{a}	35.6 ± 1.8^{a}
4	89.3 ± 1.4	56.5 ± 2.7^{b}	17.2 ± 1.8^{c}
8	88.9 ± 1.4	45.1 ± 2.7^{c}	11.5 ± 1.8^{d}

433 Different letters within the same column show significant differences among the groups (P≤0.05).

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436 Table 4. Effects of L-Carnitine (mM) on rooster sperm lipid peroxidation (M±SEM) during 0, 24
437 and 48 h of storage at 5°C.

LC concentrations	0	24	48
0	1.53 ± 0.45	3.81 ± 0.52^{b}	4.76 ± 0.49^{b}
0.5	1.50 ± 0.45	3.67± 0.52^{b}	4.78 ± 0.49^{b}
1	1.57 ± 0.45	2.15 ± 0.52^{a}	3.42± 0.49^{a}
2	1.51 ± 0.45	2.21 ± 0.52^{a}	3.38 ± 0.49^{a}

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4	1.49 ± 0.45	3.76 ± 0.52[b]	4.85 ± 0.49[b]
8	1.45 ± 0.45	3.84 ± 0.52[b]	4.91 ± 0.49[b]

438 Different letters within the same column show significant differences among the groups (P≤0.05).

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442 Table 5. Effects of L-Carnitine (mM) on rooster sperm mitochondria activity (M±SEM) during

443 0, 24 and 48 h of storage at 5°C.

LC concentrations	0	24	48
0	81.8 ± 2.5	60.1 ± 1.5[b]	13.8 ± 1.7[b]
0.5	84.2 ± 2.5	66.1± 1.5 [a]	18.4 ± 1.7 [a]
1	82.4 ± 2.5	66.3 ± 1.5[a]	18.7 ± 1.7[a]
2	85.3 ± 2.5	69.5 ± 1.5 [a]	19.2 ± 1.7[a]
4	83.1 ± 2.5	55.7 ± 1.5[c]	8.2 ± 1.7[c]
8	89.7 ± 2.5	34.1 ± 1.5[d]	2.3 ± 1.7[d]

444 Different letters within the same column show significant differences among the groups (P≤0.05).

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448 Table 6. Effects of L-Carnitine (mM) on fertility potential of rooster sperm at 24 of cool storage.

Dose of LC in extenders (mM)	0	1	2
Fertility rate (%)	64[b] (256/400)	78[a] (312/400)	80[a] (320/400)

Hatching rate (%)	56^{a} (224/400)	69^{a} (276/400)	73^{a} (292/400)

449 Different letters within the same row show significant differences among the groups (P≤0.05).

450 Number of eggs: 400 per group, number of hens: 20 per group.

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