# The capability of L-carnitine-mediated antioxidant on cock during aging: evidence for the improved semen quality and enhanced testicular expressions of GnRH1, GnRHR, and melatonin receptors MT 1/2

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**ABSTRACT** Precise natural anti-oxidative compounds have facilitated the research of infertile gametes and the development of novel bio-therapeutics, especially the molecules that are based on the reduction of oxidative stress, such as L-carnitine (**LC**). In addition to, the defect in the functioning of sperm mitochondrial and the decreasing seminal antioxidant ability due to aging, its essential role in permitting the mitochondrial import and oxidation of long chain fatty acids is worthy. Therefore, current study was designed to investigate the effects of dietary LC on semen quality, seminal antioxidant activity, and their implications for the fertility in aged cocks for 12 wk. Supplementation of the feed with two different doses of LC (50 and 150 mg/kg body weight/day) for 12 wk showed significantly increased in the reproductive activity of cock, in comparison to the control group. Seminal analysis showed that supplementation of LC significantly increased ($P < 0.05$) the sperm motility, concentration, livability, semen quality factor, seminal malondialdehyde concentration, catalase, and glutathione peroxidase activities. In addition, addition of LC significantly increased ($P < 0.05$) the plasma concentration of testosterone and prostaglandin E2 but posed no significant effect on the concentration of follicle-stimulating hormone. Furthermore, the findings of artificial insemination showed significant increased ($P < 0.05$) in the percentage of fertility in LC groups, while the percentage hatchability and mortality remained unchanged. Immunohistochemistry analysis revealed that LC significantly increased ($P < 0.05$) the testicular immunopositivity of MT1 and MT2. Moreover, the administration of LC to the aged cocks enhanced ($P < 0.05$) GnRH1 and GnRHR mRNA levels when compared with untreated cocks. The results of the present study suggest that LC treatment of aged cocks increases the seminal antioxidant enzymes and sexual hormones levels, which may improve the semen quality by increasing the expression of GnRH1 and melatonin receptors (MT1 and MT2) activities. Collectively, LC could be a suitable feed supplementation to increase reproductive activities through enhancing semen quality in aging cocks.

**Key words:** L-carnitine, antioxidant, melatonin receptors, semen quality, aging cock

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## INTRODUCTION

The insurgency of current reproductive biology investigation and assisted reproductive technologies are encouraging to provide a detailed description about the effects of metabolic natural anti-oxidative compounds supplementation on semen quality against the infertility. Especially for those that is associated with the manifestations due to aging. To date, several synthesizing supplements have been recommended to lessen the consequences of reproductive disorders, but most of these have terminated due to the numerous side-effects on other metabolic systems (Rutkowski and Grzegorczyk, 2012). In order to overcome this issue, the researchers are now searching for some natural supplements or mixture of supplements to combat against the infertility problems. Indeed, the nourishment of mitochondrial-gametes by means of nutrient supplementation has also been testified to be active in many cases (Bentov et al., 2010). In this regard, mitochondrial metabolism of both female and male gametes plays a vital role (May-Panloup et al., 2007). The L-carnitine (**LC**)


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is a biologically active stereoisomer of 3-carboxy-2-hydroxy-N, N, N-trimethyl-1-propanaminium that exists as an extremely polar and small zwitterion (Binienda and Virmani, 2003). Similarly, the metabolic pathway of LC is essential for the transportation of fatty acyl-CoA molecules into the mitochondrial matrix to start $\beta$-oxidation action that generates Adenosine triphosphate (**ATP**) for ongoing cell function (Vlies et al., 2005). Therefore, LC plays multifunctional roles in intermediary metabolism of poultry, which improves growth performance, immune responses, antioxidant activities, and semen quality (Adabi et al., 2011).

The effect of LC on semen quality is now well recognized to have an important role in proper functioning of mitochondrial oxidation, membrane integrity preservation, sperm energy balance, and apoptosis inhibition. The previous studies have shown the beneficial effects of LC on sperm quality traits specially motility and viability in different species, such as human (Garolla et al., 2005), boar (Yeste et al., 2010), stallion (Morris and Gibb, 2016), rabbit (Sarıözkan et al., 2014), rat (Abdelrazek and Oah, 2017), quail (Sarica et al., 2007), duck (Aldaraji and Tahir, 2014), and cock (Neuman et al., 2002). Additionally, the insufficiency of LC caused by aging has reduced most of the body metabolic functions especially mitochondrial performance and metabolic control (Calabrese et al., 2010). On the other hand, LC treatment has shown the improvement against the metabolic troubles caused by aging (Nicassio et al., 2017). The process of aging increases the infertility especially in spermatozoa cells because the cytoplasmic content of these cells lost during spermatogenesis. As a result, spermatozoa cells become more sensitive to oxidative stress and improper functioning of the reproductive axis (Almeida et al., 2017; Bisht et al., 2017). Thus, the Malondialdehyde (**MDA**) concentration, Catalase (**CAT**) and Glutathione Peroxidase (**GSH-PX**) activities, and natural anti-oxidative compounds (i.e., LC) possess key functions to protect sperm against the oxidative stress by preventing the production of free radicals (Martínezpáramo et al., 2012).

Melatonin receptors (**MT1** and **MT2**) are the focal sites of male reproductive tract (Gonzálezarto et al., 2017). These have great functional features such as scavenging free radicals, a broad-spectrum of antioxidant in order to reduce the reactive oxygen species (**ROS**) and lipid peroxidation (**LP**) (Garolla et al., 2005), enhance biosynthesis of hormones e.g., gonadotropins and testosterone (Li and Zhou, 2015), increase the functioning of Leydig and Sertoli cells (Yu et al., 2018), and regulate the hypothalamus-pituitary-gonadal axis (Alvarado et al., 2015). But as male gets old, the expression of these receptors is decreased (Sánchez-Hidalgo et al., 2009). Thus, the reduction of melatonin receptors functions is associated with the aging process and causes infertility but this fertility can be enhanced through the administration of LC (Esposti et al., 1994). Indeed, both LC and melatonin receptors have a similar importance for the enhanced scavenging activity that reduces oxidative stress (Shaker et al., 2009). In addition, GnRH is an appetite hypothalamic act as a regulator of gonadal function that induces the biosynthesis and the release of pituitary gonadotropins, which in turn promotes the steroidogenesis and spermatogenesis (Garolla et al., 2005). Li et al. (1997) reported that melatonin receptors administration in aged male rats enhanced the GnRHR mRNA levels and reversed the influence of aging.

Our objective was to examine the role of administrating LC for improving the semen quality, seminal antioxidant enzymes activity, fertility, and expression of some candidate reproduction genes in aging cock was investigated. More precisely, the effect of LC on physical semen traits was analyzed for 12 wk and plasma sexual hormones as well as seminal antioxidant enzymes were quantified. Fertility in male cock was also examined during the whole experimental periods. Finally, the mRNA expression for GnRH1 and GnRHR genes was detected and analyzed for testicular immunolocalization for MT1 and MT2. This study was keenly directed on finding new insights in breeding feed model in order to improve the reproductive activity in aging cocks.

## MATERIALS AND METHODS

### Ethics Statement

The study was conducted at the Poultry Farm under the Laboratory of Animal Genetics, Breeding and Reproduction, College of Animal Science and Technology, Huazhong Agricultural University, Wuhan, P. R. China and carried out in according to the Chinese Animal Welfare regulations. The samples collection protocol was followed by the guidelines established for the Care and Use of Laboratory Animals of the Standing Committee of Hubei People's Congress (No. 5), approved by the Animal Care Committee of Hubei Province, P.R. China, and the Ethics Committee of Huazhong Agricultural University, Wuhan, P. R. China.

### Bird Management and Experimental Design

For the present experiment, a total of 36 aged crossbreed cocks of same ages (55 wk) and similar weights ($2.2 \pm 0.15$ kg) were selected. The experimental roosters were divided into three groups, with 12 cocks in each group and kept individually in cages ($70 \times 60 \times 75$ cm) inside the enclosed chicken farm. All cocks were kept under same management systems during the experimental periods and fed with standard isocaloric and isonitric diet (2700 kcal/kg and 13% protein) and drinking water was provided ad-libitum. The control group (LC-0) was fed on the standard diet without LC supplementation, where as the second (LC-50) and third (LC-150) groups were fed on the same standard diet

with 50 and 150 mg/kg body wt/day for 12 wk of experimental period, respectively.

### Semen Collection and Evaluation

Cocks were trained to collect the semen 2 wk before starting the collection and then semen samples were collected twice per week for 12 wk from each cock at early morning before feeding using the manual abdominal massage method, according to Burrows and Quinn (1937) . The following parameters were determined immediately after the semen collection; semen volume using a micropipette, pH with a pH indicator paper, weight of ejaculate by difference of weight between empty and sample's tube, sample viscosity and color were macroscopically estimated (slight, moderate and high). Additionally, sperm concentration was measured by using the spectrophotometer, percentage of viable sperm by using aniline blue stain method (Bilgili and Renden, 1984). Individual motility grade and percentage of mass motility were also evaluated (Blesbois et al., 2008) and then sperm quality factor (**SQF**) was calculated according to the mathematical equation described by Liu et al. (2008).

### Artificial Insemination and Fertility Calculation

To verify the impact of supplementary LC on improving semen quality and male fertility, a total of 60 hens of same age (47 wk) were divided into 3 groups (20 hens in each group). The first group was artificially inseminated by the semen of LC-0 group. Likewise, the second and third groups were inseminated by LC-50 and LC-150 groups, respectively. Artificial insemination was applied once per week, the eggs were collected for incubation and percentages of fertility, mortality, and hatchability. The fertility experiment was done in three replicates. The average of fertile eggs was determined at the 6th d of egg incubation using light detection and data were calculated as the percentage of fertile eggs to total eggs.

### Measurement of Plasma Testosterone, FSH and PGE2

Blood samples were collected from a superficial venipuncture of a wing vein of each cock twice per month at the third month of the experimental period ($n = 24$) using sodium citrate as an anticoagulant. Then, the samples were centrifuged at 3000 rpm for 10 min and the plasma was stored at −20 °C for subsequent analysis. Testosterone, follicle-stimulating hormone (**FSH**), and PGE2 concentrations were detected in 24 plasma samples from each group using an enzyme-linked immune sorbent assay (ELISA) according to the manufacturer's instructions (Njjcbio, China) as described by Hao (2006).

### Measurement of Seminal MDA Concentration, CAT, and GSH-PX activity

Seminal fluid was collected twice per month at the third month of the experimental period ($n = 24$) using two centrifugations, first at 16,000 rpm for 20 min at 4°C to separate the seminal fluid from sperm then upper layer of fluid was collected and second centrifugation was done at 16,000 rpm for 10 min at 4°C to remove any remaining sperm (Thacker et al., 2011) and finally stored at −80°C for antioxidant enzyme analyses. MDA concentration, CAT and GSH-PX activity were measured in 24 seminal fluid samples using commercial kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, P. R. China). The commercial kit based on thiobarbituric acid reaction was used to measure MDA concentration in the seminal plasma as indices of LP in the semen samples. Briefly, after mixing 0.2 mL of homogenate with the trichloroacetic acid and centrifugation, the obtained supernatant was mixed with thiobarbituric acid. After this, the absorbance of the product formed by MDA and the thiobarbituric reaction was determined at 532 nm using a spectrophotometer (Placer et al., 1966). Additionally, CAT activity was determined by ammonium molybdate. The resultant yellow turbidity was measured in terms of OD value at 405 nm using a spectrophotometer as CAT activity (Goth, 1991). The activity of GSH-PX was measured with dithio-dinitro benzoic acid reaction and produced 5-thio-dinitro benzoic acid anions with relatively stable yellow color that was quantified by measuring absorbance at 412 nm with a spectrophotometer (Hafeman et al., 1974).

### Immunohistochemical Localization and Analysis of Testicular MT1 and MT2

A total of 15 cocks (five in each group) were used to obtain testicular immunohistochemistry analyses at the 12th wk of experimental period. The melatonin receptors (MT1 and MT2) protein in testis were localized by immunostaining following the protocol of Huang et al. (2014) and Ansari et al. (2016). In brief, the tissue sections were incubated overnight at 4°C with primary rabbit polyclonal MT1 antibody (Cat: 17,172–1-AP) and rabbit polyclonal MT2 antibody (Cat: 20,809–1-AP) according to the manufacturer instructions (Proteintech, Wuhan Sanying Biological Technology Co. Ltd, China). Then, after washing with PBS, the tissue sections were incubated at room temperature for 2 h with secondary goat anti-rabbit antibody (Cat: SA00001–2, http://www.ptgcn.com) for both MT1 and MT2. Again after washing with PBS, the tissue sections were immersed in diaminobenzidine solution to obtain a brownish yellow color. The hematoxylin was used as

**Table 1.** Information of qRT-PCR primers.

| Genes | Primers | Sequences | Base | GC% | Product length | n.mol/OD | MW |
|---|---|---|---|---|---|---|---|
| GnRH1 | Forward | 5′ TGGTCTTATGGCCTGCAACC 3′ | 20 | 50.00 | 127 | 5.4 | 6084.0 |
| | Reverse | 5′ GGTAAGAGCCAGGGCATTCA 3′ | 20 | 55.00 | | 5.3 | 6191.1 |
| GnRHR | Forward | 5′ TCACCGTCGTCATCAGCCT 3′ | 19 | 57.89 | 155 | 5.8 | 5699.7 |
| | Reverse | 5′ GGTGACGGTGTGGAAGAGG 3′ | 19 | 63.16 | | 5.5 | 6014.0 |
| $\beta$-actin | Forward | 5′ ACCCCAAAGCCAACAGAGAG 3′ | 20 | 55.00 | 105 | 5.4 | 6098.0 |
| | Reverse | 5′ CAGAGGCATACAGGGACAGC 3′ | 20 | 60.00 | | 5.3 | 6185.1 |

counter stain for staining nuclei. Then, the tissue sections were cleaned in xylene and mounted with DPX. A Zeiss light microscope was used for taking photos. The serial sections were examined under a light microscope (BH-2; Olympus, Japan) with a digital camera (DP72; Olympus). The distribution and expression level of different proteins were measured in high-power fields selected at random. All of the images were taken using the same microscope and camera. Image-Pro Plus 6.0 software (Media Cybernetics, USA) was used to calculate the mean density for positive staining. Background lighting of images was calibrated to measure the integrated optical density (**IOD**) per area, which was used to determine the concentration of antigen of interest. The results of IOD were expressed as a percentage of staining intensity and the area of staining.

### Detection of mRNA Expression of GnRH1 and GnRHR Using qRT-PCR Analysis

The testicular tissues ($n = 5$) were immediately collected after slaughtering, cut into small pieces and kept into liquid nitrogen for a short time. After that, the frozen samples were homogenized using ultrasonic homogenizer (UP100H, Hielscher, Germany). Total RNA for qRT-PCR analysis was extracted from homogenized tissue samples using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions (Das et al., 2010). The quantity and quality of RNA were detected by NanoDrop (ND2000, Thermo Fisher Scientific, Waltham, MA, USA) spectrophotometer and gel electrophoresis, respectively. Reverse transcription of first strand complementary DNA (cDNA) synthesis was performed using TAKARA Bio Inc., Japan as per manufacturer's instruction. The SYBR Green, real-time PCR master mix (Toyobo Co., Ltd., Osaka, Japan) for the qRT-PCR reaction was used to detect the GnRH1 and GnRHR expression, following the manufacturers' instructions (Mehta et al., 2010). The primers were designed from NCBI primer blast, and primers were checked using OLIGO7 software (Molecular Biology Insights) in accordance with the sequences of the corresponding gene's mRNAs from Gene Bank. A list of primers of selected genes for the qRT-PCR experiment is shown in Table 1. Chicken $\beta$-actin gene was used as a housekeeping gene in qRT-PCR expression experiments that acted as a control gene for normalization of cDNA loading differences in this experiment. The qRT-PCR reaction contained 5.0 $\mu$L of SYBR Green real-time PCR master mix (Toyobo Co., Ltd., Osaka, Japan), 0.2 $\mu$L of forward and reversed primer each, 1.0 $\mu$L of cDNA, and 3.6 $\mu$L of RNase free $dH_2O$. The qRT-PCR reaction was performed on a Bio-Rad thermal cycler, CFX-96, real-time system as follows: the single cycle of denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 15 s, annealing at 59°C for 20 s, and extension at 72°C for 15 s.

### Statistical Analysis

The data were analyzed by the GLM procedure of SAS (SAS Institute Inc., 2002, Cary, NC, USA, version 9). Results were expressed as Least Square Means (LSM ± SEM) and Duncan's multiple range test were done to compare the differences between means. All data were evaluated for conformity to a normal distribution with the Kolmogorov-Smirnov (K-S) test and all percentage data were normalized through arcsine transformation. $Y_{ij}=\mu+T_i+e_{ij}$ statistical model was used to analyze semen quality traits, in where, $Y_{ij}$ is observation; $\mu$ is the parameters mean; $T_i$ is the effect for LC doses (LC-0, LC-50, and LC-150), and $e_{ij}$ is experimental error assumed to be randomly distributed ($\sigma 2 = 0$). The 4 biological and 3 technical replications per sample were used for qRT-PCR expression analysis. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression according to Livak and Schmittgen (2001) and Livak (2008). Comparisons between expression values of qRT-PCR datasets were also made using *T*-test.

## RESULTS

### Effects of LC on Semen Quality and Fertility

The results of dietary administration of LC for 12 wk on semen quality of aging cocks are shown in Table 2. The ejaculate volume and weight were significantly ($P < 0.05$) increased in LC-150 group compared with LC-50 and LC-0 groups. Moreover, the highest average for mass motility and individual motility traits ($P < 0.05$) was also found in LC-150 group (37.61 ± 3.77 and 3.43 ± 0.06, respectively). Sperm concentration and livability were significantly increased ($P < 0.05$) in LC-150 group compared with LC-50 and LC-0 groups. Significantly ($P < 0.05$) increased SQF was found in LC-150 group then the LC-50 and LC-0 groups. No significant effect of LC was found on $P^H$, color, and viscosity of semen among all experimental groups (Table 2).

**Table 2.** Semen quality characteristics in different L-carnitine (LC) treatment groups in aging cocks.

| Parameters | Treatments (LSM ± SEM) | | | *F* value | *P* value |
|---|---|---|---|---|---|
| | LC-0 | LC-50 | LC-150 | | |
| Ejaculate volume (mL) | 0.22 ± 0.04$^{c}$ | 0.26 ± 0.04$^{b}$ | 0.29 ± 0.04$^{a}$ | 12.26$^{***}$ | 0.0001 |
| Color grade | 1.23 ± 0.034 | 1.27 ± 0.034 | 1.25 ± 0.032 | 0.57 | 0.6839 |
| Viscosity grade | 2.29 ± 0.05 | 2.22 ± 0.05 | 2.19 ± 0.05 | 0.73 | 0.4822 |
| Ejaculate weight (mg) | 0.10 ± 0.02$^{b}$ | 0.14 ± 0.02$^{a}$ | 0.16 ± 0.02$^{a}$ | 88.58$^{***}$ | 0.0001 |
| PH grade | 7.24 ± 0.04 | 7.20 ± 0.04 | 7.16 ± 0.04 | 1.15 | 0.3189 |
| Mass motility (%) | 44.02 ± 3.10$^{c}$ | 62.57 ± 3.10$^{b}$ | 69.28 ± 3.02$^{a}$ | 148.95$^{***}$ | 0.0001 |
| Individual motility grade | 2.27 ± 0.97$^{c}$ | 3.06 ± 0.97$^{b}$ | 3.38 ± 0.96$^{a}$ | 69.89$^{***}$ | 0.0001 |
| Sperm concentration ($10^{9}$/mL) | 3.15 ± 0.59$^{c}$ | 3.72 ± 0.50$^{b}$ | 4.85 ± 0.59$^{a}$ | 83.28$^{***}$ | 0.0001 |
| Live sperm (%) | 66.88 ± 5.60$^{c}$ | 69.25 ± 5.61$^{b}$ | 74.84 ± 5.56$^{a}$ | 49.39$^{***}$ | 0.0001 |
| Semen quality factor (SQF) | 68.34 ± 8.27$^{b}$ | 74.71 ± 8.30$^{b}$ | 112.46 ± 8.03$^{a}$ | 58.64$^{***}$ | 0.0001 |

LSM = Least square means; SEM = standard error of means; "$^{***}$" means significant at $P < 0.001$; $^{a, b, c}$different letters indicated significantly differences between means. Cocks/group $n = 12$; Ejaculate/cock $n = 24$.

**Table 3.** Descriptive statistics and correlation analysis among semen quality traits in aging cocks.

| Traits | Mean | SEM | Min. | Max. | EV | EW | CON | MM | LS | SQF |
|---|---|---|---|---|---|---|---|---|---|---|
| EV | 0.33 | 1.31 | 0.10 | 23.00 | 1.00 | | | | | |
| EW | 0.10 | 0.05 | 0.04 | 0.50 | 0.96$^{***}$ | 1.00 | | | | |
| CON | 3.68 | 0.79 | 0.20 | 5.49 | − 0.23$^{***}$ | − 0.26$^{***}$ | 1.00 | | | |
| MM | 26.84 | 10.56 | 3.80 | 60.00 | 0.12 $^{**}$ | 0.09 | 0.26$^{***}$ | 1.00 | | |
| LS | 65.00 | 7.56 | 20.00 | 80.00 | 0.13$^{**}$ | 0.19$^{**}$ | 0.20$^{***}$ | 0.48$^{***}$ | 1.00 | |
| SQF | 63.30 | 40.79 | 14.19 | 214.80 | 0.54$^{***}$ | 0.44$^{***}$ | 0.76$^{***}$ | 0.45$^{***}$ | 0.42$^{***}$ | 1.00 |

SEM = standard error of the mean; EV = ejaculate volume (mL); EW = ejaculate weight (mg); CO$n$ = sperm cell concentration × $10^{9}$; MM = mass motility %; LS = live sperm %; SQF = semen quality factor; "$^{**}$" & "$^{***}$" means significant at $P$ <0.01 and $P$ <0.001, respectively.

The correlation among semen quality traits in experimental groups are illustrated in Table 3. The trend of coefficients was generally from very low to high and the r values were ranged from = 0.09 to 0.94. A high positive correlation ($r = 0.94$) was found between ejaculate volume and weight, where as between sperm concentration and mass motility showed moderate ($r = 0.46$) and live sperm showed low ($r = 0.20$) correlation. Likewise, SQF also showed positive correlation with sperm concentration ($r = 0.76$), mass motility ($r = 0.45$), and live sperm ($r = 0.42$). On the other hand, sperm cell concentration showed negative correlation with ejaculate volume ($r$ = -0.04) and ejaculate weight ($r$ = -0.16).

Reproductive performances in hens inseminated by semen from different experimental aging cocks among the three groups are illustrated in Table 4. The significantly ($P < 0.05$) highest fertility percentage was found in LC-150 group compared to LC-50 and LC-0 groups. On the other hand, there was no significant difference found between the percentage of hatchability and mortality in all groups (Table 4).

## *Effects of LC on Blood Hormones (Testosterone, FSH, and PGE2)*

The results for the effects of dietary administration of LC on the plasma concentration of testosterone, FSH, and prostaglandin E2 (PGE2) of aging cocks are summarized in Table 5. The concentration of testosterone was significantly increased ($P < 0.05$) in the both of LC-50 and LC-150 compared with LC-0. The highest level was in LC-150 compared with LC-0 and LC-50 groups. However, the highest concentration of PGE2 was measured in LC-50 compared with LC-150 and LC-0 groups. On the other hand, administration of LC didn't show any significant effect on the blood content of FSH.

## *Effects of LC on Seminal MDA Concentration, CAT, and GSH-PX Activity*

The results of seminal MDA concentration, CAT, and GSH-PX activity of three groups are illustrated in Table 6. The highest average ($P < 0.05$) for seminal MDA concentration was recorded in LC-0 compared with LC-50 and LC-150 groups. On the other hand, seminal CAT activity was significantly increased in both of LC-50 and LC-150 groups compared with LC-0. As far as the seminal GSH-PX activity was concerned, the highest level was recorded in LC-50 compared with LC-150 and LC-0 groups (Table 6).

## *Effects of LC on Testicular Immunolocalisation MT1 and MT2*

In the immunostaining, it was observed that immune positive signals of MT1 and MT2 were increased in

**Table 4.** Reproductive performances in hens inseminated by semen from different experimental aging cocks.

| Parameters | Treatments (LSM ± SEM) | | | *F* value | *P* value |
|---|---|---|---|---|---|
| | LC-0 | LC-50 | LC-150 | | |
| Fertility rate (%) | 69.19 ± 2.47$^{b}$ | 74.39 ± 2.47$^{b}$ | 84.58 ± 2.47$^{a}$ | 10.03$^{**}$ | 0.003 |
| Hatchability (%) | 88.34 ± 1.22 | 88.94 ± 1.22 | 89.96 ± 1.22 | 0.45 | 0.460 |
| Embryonic mortality (%) | 11.65 ± 1.22 | 11.05 ± 1.22 | 10.03 ± 1.22 | 0.45 | 0.730 |

LSM = Least square means; SEM = standard error of means; "**" means significant at $P < 0.01$; $^{a, b, c}$different letters indicated significantly differences between means. Cocks/group $n = 12$; Hens/group $n = 20$; Insemination replicates $n = 3$.

**Table 5.** Sexual hormones status in blood plasma of different level L-carnitine (LC) treatment groups in aging cocks.

| Parameters | Treatments (LSM ± SEM) | | | *F* value | *P* value |
|---|---|---|---|---|---|
| | LC-0 | LC-50 | LC-150 | | |
| T (ng/mL) | 20.19 ± 4.98$^{b}$ | 33.93 ± 4.98$^{a,b}$ | 45.25 ± 4.98$^{a}$ | 6.34$^{**}$ | 0.010 |
| FSH (mIU/mL) | 105.32 ± 25.71 | 127.40 ± 25.71 | 141.36 ± 25.71 | 0.61 | 0.610 |
| PGE2 (ng/mL) | 0.83 ± 0.31$^{b}$ | 1.84 ± 0.31$^{a}$ | 1.55 ± 0.31$^{a}$ | 3.69$^{*}$ | 0.020 |

LSM = Least square means; SEM = standard error of means; T = Testosterone; FSH = Follicle stimulating hormone; PGE2 = Prostaglandin E2; "*" and "**" means significant at $P < 0.05$ and $P < 0.01$; $^{a, b, c}$different letters indicated significantly differences between means. Samples/group $n = 24$.

**Table 6.** Antioxidant parameters in seminal fluid of different level L-carnitine (LC) treatment groups in aging cocks.

| Parameters | Treatments (LSM ± SEM) | | | *F* value | *P* value |
|---|---|---|---|---|---|
| | LC-0 | LC-50 | LC-150 | | |
| MDA (nmol/mL) | 2.75 ± 0.27$^{a}$ | 2.04 ± 0.27$^{a,b}$ | 1.31 ± 0.27$^{b}$ | 6.91$^{**}$ | 0.007 |
| CAT (U/mL) | 38.10 ± 12.15$^{b}$ | 41.03 ± 12.15$^{b}$ | 50.81 ± 12.15$^{a}$ | 6.14$^{**}$ | 0.010 |
| GSX-PX (U/mL) | 21.32 ± 6.99$^{b}$ | 26.26 ± 6.99$^{a}$ | 24.65 ± 6.99$^{a}$ | 5.43$^{**}$ | 0.009 |

LSM = Least square means; SEM = standard error of means; MDA = Malondialdehyde; CAT = Catalase; GSX-PX = Glutathione Peroxidase; "**" means significant at $P < 0.01$; $^{a, b, c}$ different letters indicated significantly differences between means. Samples/group $n = 24$.

the seminiferous tubules in both LC-50 and LC-150 groups and almost all seminiferous tubules were undergoing complete spermatogenesis (Figure 1, B and C) compared with LC-0 (Figure 1, A). The LC enhanced the spermatogenesis, active Sertoli cells inside tubules, and Leydig cells among the tubules. It can be observed from the figure that the MT1 and MT2 protein was localized in the spermatogonia cells (Figure 1A-C, indicated by arrows). MT1 and MT2 were strongly concentrated in the spermatogonia cells, spermatozoa, and Sertoli cells in both of LC-50 and LC-150 compared with LC-0. Generally, immunopositivity for MT1 and MT2 were appeared as dark brown spots in intracytoplasmic of spermatocyte inside seminiferous tubules, while Leydig cells were appeared with less intensity. The highest immunopositive signals of MT1 and MT2 were appeared in LC-150 and LC-50, respectively (Figure 1A-C). These changes were significantly attenuated in the testicular tissues of LC-50 and LC-150 group compared to the LC-0 group. Significant differences ($P < 0.05$) in the IOD of both of LC-50 and LC-150 groups were also observed in the cocks compared to the LC-0 (Figure 1D). The highest average of IOD for MT1 was recorded for LC-150, although the average of IOD was the highest for the LC-50 as shown in Figure 1D.

## *Effects of LC on mRNA Expressions of GnRH1 GnRHR*

The relative mRNA expression of GnRH1 and GnRHR genes were compared using qRT-PCR in the testicular tissues between LC-0 and LC-150 groups in aging cocks following $2^{-\Delta\Delta CT}$ method (Figure 2). The PCR amplification showed for specific DNA fragments of 127 bp for GnRH1 and 155 bp for GnRHR (Figurefig. 2a). The qRT-PCR data were normalized by $\beta$-actin housekeeping gene for each sample. It was seen that the expression profiles were very similar for these two genes, while gene GnRH1 expressed approximately double expression then the GnRHR (Figure 2, b and c). The genes GnRH1 and GnRHR showed significantly ($P < 0.01$) higher expression in the LC-150 group than the LC-0 group.

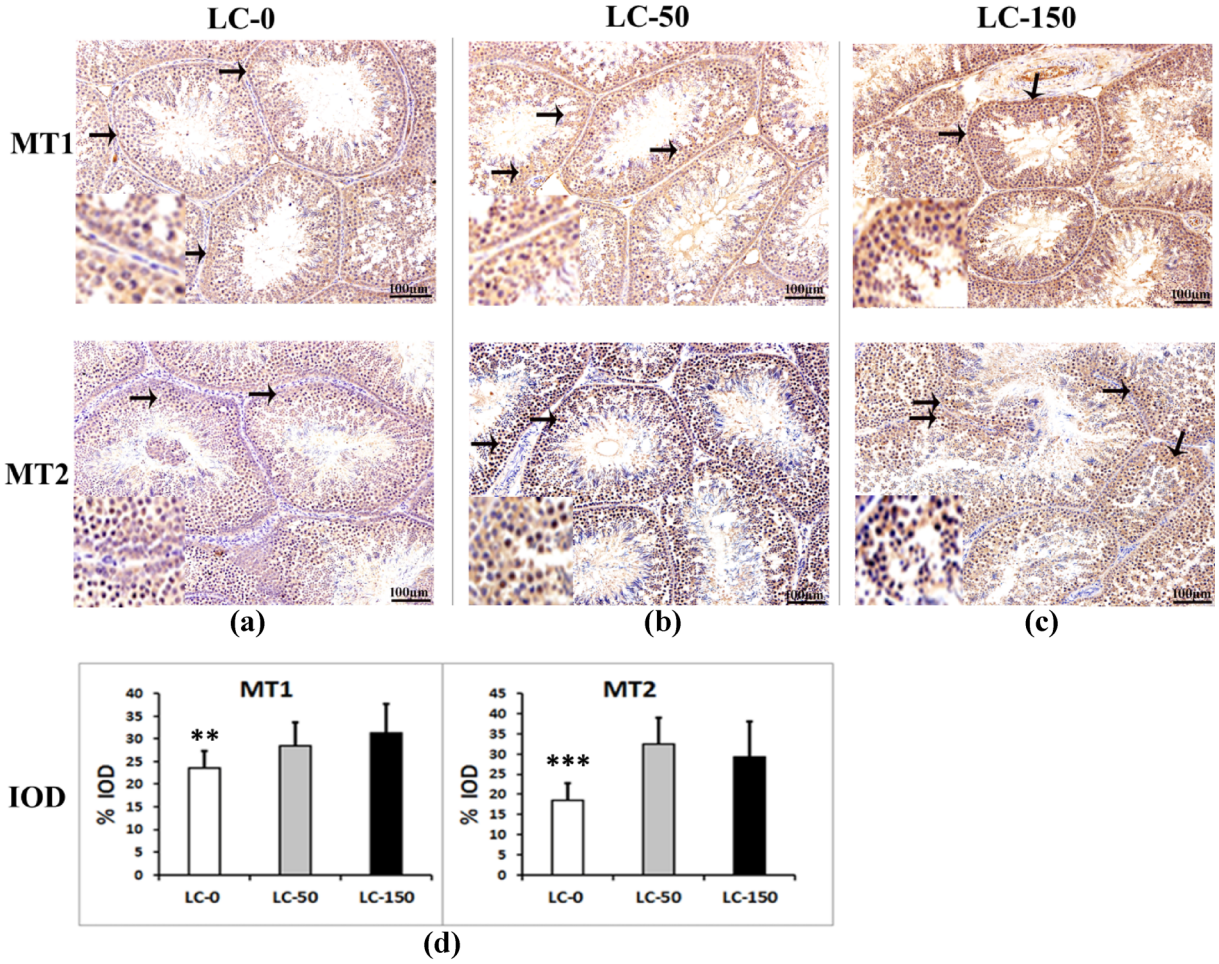


**Figure 1.** Immunohistochemistry of melatonin receptors in the testicular sections of cocks. The top raw (a, b, and c) shows melatonin receptors 1 (MT1), the middle raw (d, e and f) shows the melatonin receptors 2 (MT2), and the bottom row (g and h) shows the percentage of integrated optical density (IOD) for MT1-immunopositive and MT2-immunopositive, respectively. The spermatogenic cells show immunopositive reaction (arrows) in control (LC-0) and L-carnitine (LC) administration (LC-50, and LC-150) groups. The MT1-immunopositive signals shows high expressed in LC-150, moderate in LC-50, and low in LC-0, whereas MT2-immunopositive signals shows high expressed in LC-50, moderate in LC-150, and low in LC-0. ("**" & "***" means significant at $P < 0.01$ and $P < 0.00$, respectively. $P < .05$; Scale bar = 100 $\mu$m; $n = 5$).

## DISCUSSION

In the aging, the semen characteristics are decreased due to the dramatic increase in seminal LP activity, which is a candidate factor for decreasing sperm motility due to the presence of high concentrations of long-chain fatty acid into seminal plasma and thus become substandard sperm in fertility, viability, and lifetime. In addition, LP activity may be correlated with the decreased seminal antioxidant enzymes activity and accumulation of the both peroxides and free radicals which are further available for activation of LP, ROS, and oxidative stress (Almeida et al., 2017; Bisht et al., 2017). The peroxidation process is functionally based on sperm structural alterations, especially membrane and acrosomal site as well as the DNA damage of the spermatozoa by leakage that also constituents intracellular spermatic. To prevent or decrease these effects, supplementation of LC can improve the semen quality, and antioxidant enzyme activities (Garolla et al., 2005; Sarica et al., 2007; Yeste et al., 2010). The results of the present study showed that the response to the LC treatment was detectable after 4 wk from starting the experiment. In our study, the LC administration continued for 12 wk, which is presumed to be adequate for investigating the response of LC in the rooster's spermatogenesis process. The duration of spermatogenesis in domestic birds was reported to range from 11 to 15 d (Lin and Jones, 1992); it is four times much faster than that (45.8 to 75 d) in mammals (Roosen-Runge 1977). The results of the current experiment have demonstrated a positive effect of LC on spermatozoa quality by enhancing the seminal enzymatic, which protects the structures and functions of spermatozoa efficiently against the oxidative stress. Addition of the LC in diet improved the sperm quality parameters (motility, viability, and concentration), acrosomal abnormality, plasma membrane integrity, and promoted male fertility after duration of treatment (Neuman et al., 2002; Adabi et al., 2011; Morris and Gibb, 2016). Thus, it may be a relative enhancer of sperm fertilizing capacity during an aging stage. Analysis of various factors for seminal traits such

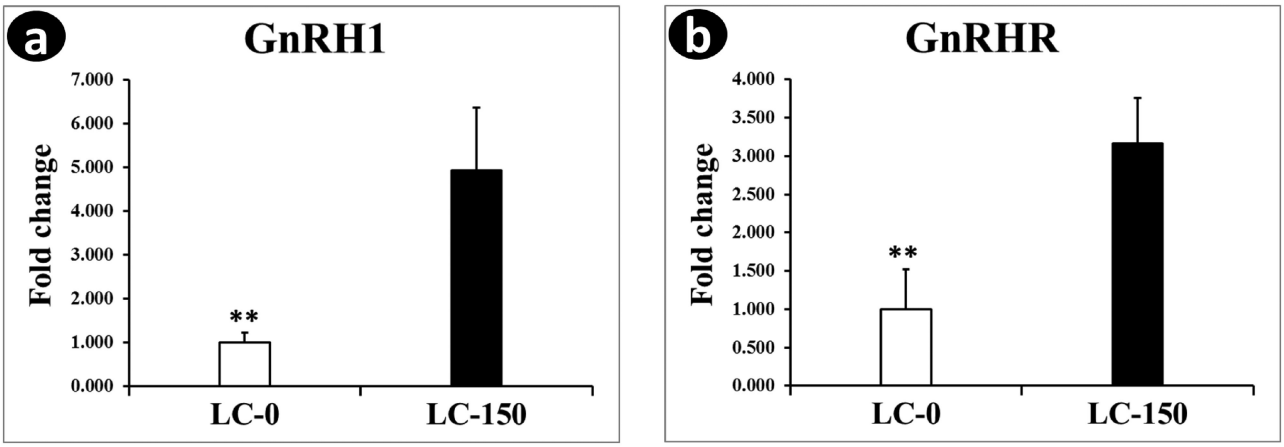


**Figure 2.** The relative expression of control and treatment group (LC-150) of aged cock by qRT-PCR. (a) Relative expression of GnRH1 gene and (b) relative expression of GnRHR gene. All samples were normalized by $\beta$-actin (housekeeping) gene. Significant at $P < 0.05$, based on assuming equal variances of $t$-test. Error bar represents standard error of the mean (SEM). ("**" means significant at $P < 0.01$; $n = 5$)

as ejaculate volume, mass and individual motility, livability, and concentration are important for the determination of fertility of semen in artificial insemination. In the present study, supplementation of LC revealed a significant difference between the LC treatments groups compare to control group regarding these parameters. The beneficial effect of LC on semen is due to its powerful anti-oxidative nature. Therefore, LC supplementation has been suggested for the defense functioning of sperm against the LP in order to enhance the semen quality in different species such as human (Garolla et al., 2005), stallion (Stradaioli et al., 2004), bull (Abdel-Khal et al., 2015), drake (Aldaraji and Tahir, 2014), boar (Yeste et al., 2010), quail (Sarica et al., 2007), and cock (Neuman et al., 2002). The role of LC for the treatment of spermatogenesis, disorders caused by aging that renders the sperm very protectable from LP, is also well documented (Almeida et al., 2017). Therefore, the treated groups (LC-50 and LC-150) showed increased sperm quality than the control group, which leads to the enhanced fertilizing ability of sperm in storage tubules of the oviduct. Additionally, our results also showed that percentage of fertility increased in LC groups due to the improvement in seminal quality. Likewise, the plasma concentration of testosterone and PGE2 were increased in LC groups due to functional improvement of Leydig cells by releasing GnRHR (Estienne, 2014).

The results of the present study also showed that MDA concentration was decreased due to LC supplementation, which is positively correlated with the seminal LP. On the other hand, the seminal CAT, and GSH-PX activity was increased in LC groups as a powerful marker of seminal antioxidant efficiency. Consequently, it protected the sperm in the epididymis as well as in oviduct during storage through less formation of ROS (Rui et al., 2017). It has been reported that there existed negative correlation among semen traits such as motility, viability, and normality with seminal MDA concentration (Safa et al., 2016). The seminal concentration of MDA and CAT, GSH-PX activity are very important indicators of sperm function as well as sperm fertilizing capacity because these measure the sperm membrane stability (Partyka et al., 2012). Moreover, our results reported that supplementation of LC significantly improved the plasma concentration of T, FSH, and PGE2 due to the altering responses of pituitary-gonadal axis (Shaker et al., 2009). Additionally, that improvement may be referred to the enhanced GnRH1 and GnRHR genes expression as well as the positive pushed feedback for hypothalamic-hypophyseal-testes axis, which increased the steroidogenesis and secretion of sex hormones. These results are aligned with the findings of previous studies conducted on similar fields (Ahmed et al., 2013; Genazzani et al., 2017), and showed that the increase in GnRH expression due to the high level of testosterone and FSH as well as LC treatment significantly enhanced the hypothalamus GnRH1 levels. The MTs regulates the Sertoli cell metabolism and its activists, thus it may affect all spermatogenesis traits especially the sperm motility and viability (Rocha et al., 2014). Not only this, it influences the germ cell proliferation and Sertoli cells, and regulates the hypothalamus–pituitary–gonadal to GnRH secretion (Viguie et al., 1995). Thus, our results reported the increased plasma FSH content in the both of LC-50 and LC-150 compared with LC-0, which, in turn, regulates the germ cell proliferation. In the current study, LC treatment also improved the relative mRNA expression of GnRH1 and GnRHR in the testicular tissues. The current findings are in agreement with the previous study which revealed an improvement in testicular immunopositive signals of caspase-3, decreasing of MDA concentration, and increased of GSH activity under the LC administration against cytotoxic of anticancer drug (Elgawish and Abdelrazek, 2014). It is well known that GnRHR, specifically binding with T and FSH forms a complex, allows it binding to regulate of the hypothalamus–pituitary-gonadal function, and therefore plays a critical role in the fertility (Genazzani et al., 2017).

It can be concluded that improvement of semen quality traits, especially on motility, viability, and SQF might be attributed to the prevention of excessive production of free radicals by means of abundant antioxidant produced due to the potential ability of LC. The LC supplementation enhanced seminal antioxidant activity and prevents the MDA production. In addition, it increased the activities of sexual hormones, ultimately enhanced fertility. Furthermore, LC promotes MT1, MT2, GnRH1, and GnRHR genes expression in testicular tissues. Thus, it may protect the spermatozoa during the storage in epididymis and oviduct, which enhances the fertility in aging cock. Collectively, LC could be a suitable feed supplementation to increase reproductive activities through enhancing semen quality in aging cocks.

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## CONFLICT OF INTEREST

The authors declare that there are no conflict of interest.

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