

Carnitine Supplementation Modulates High Dietary Copper-Induced Oxidative Toxicity and Reduced Performance in Laying Hens

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Abstract This experiment was conducted to evaluate the effects of L-carnitine on performance, egg quality and certain biochemical parameters in laying hens fed a diet containing high levels of copper proteinate. Forty-eight 42-week-old laying hens were divided into four groups with four replicates. The laying hens were fed with a basal diet (control) or the basal diet supplemented with either 400 mg carnitine (Car)/kg diet, 800 mg copper proteinate (CuP)/kg diet or 400 mg carnitine + 800 mg copper (Car+CuP)/kg diet, for 6 weeks. Supplemental CuP decreased feed consumption ($p<0.01$), feed efficiency and egg production ($p<0.001$), as compared to control. The combination of Car and CuP increased ($p<0.001$) egg production and feed efficiency as compared to CuP. The activities of alanine aminotransferase ($p<0.05$) and alkaline phosphatase ($p<0.01$) were increased, while lactate dehydrogenase activity was decreased ($p<0.001$) by supplemental CuP and Car+CuP. Supplemental CuP caused an increase in plasma malondialdehyde ($p<0.01$) and nitric oxide levels ($p<0.05$). In the Car+CuP group, this increase was observed to have been reduced significantly ($p<0.05$). Furthermore, Car+CuP increased ($p<0.05$) glucose level. These results indicate that the carnitine and copper combination may prevent the possible adverse effects of high dietary copper on performance and lipid peroxidation in hens.

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Abbreviations

Cu	Copper
Car	Carnitine
CuP	Copper proteinate
MDA	Malondialdehyde
NO	Nitric oxide
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ALP	Alkaline phosphatase
LDH	Lactate dehydrogenase
GGT	Gamma-glutamyl transpeptidase
Ca	Calcium
Pi	Inorganic phosphor
Mg	Magnesium
H&E	Hematoxylin–eosin
SEM	Standard error mean
H ₂ O ₂	Hydrogen peroxide

Introduction

In the organism, copper (Cu) is found mainly in the liver and also in a variety of cells and tissues, although at low levels. Copper also exists in the structure of some major enzymes (cytochrome-c oxidase, tyrosinase, *p*-hydroxyphenylpyruvate hydrolase, dopamine-beta-hydroxylase, lysyl oxidase and copper, zinc superoxide dismutase), functioning as a cofactor [1, 2]. It is advised by the National Research Council [3] that copper be added at a rate of 2.5 mg/kg to laying hen diets and at a rate of 8 mg/kg to broiler chicken diets. However, Cu can also be used at higher levels for growth stimulation and therapeutic purposes [4–6]. In such a case, the organic (proteinate and amino acid chelates) and inorganic (copper sulphate, copper oxide) forms of copper are used; yet, it is known that the organic forms of copper have better absorption and bioavailability properties [7]. It has been reported that, in poultry, 100–250 mg/kg of organic/inorganic copper increases performance and exhibits antioxidative effect [1, 5, 6, 8, 9], while exceeding levels affect performance adversely and induce oxidative effect [8, 10, 11]. The susceptibility of animals to the intake of high levels of Cu varies among species. While sheep are very susceptible to high levels of Cu; poultry, rodents and pigs are less susceptible [1]. It has been reported that, in the event of its intake by the organism at high levels, Cu leads to the generation of hydroxyl radicals, which induce major oxidative effect in biological systems, by either reacting with hydrogen peroxide (Haber–Weiss reaction) or catalyzing the reaction between super oxide radicals and water (Fenton-type reaction) [1, 12]. Furthermore, oxygen-derived free radicals that may be generated in the organism are inactivated through enzymatic and nonenzymatic defence mechanisms [13]. In recent years, the antioxidant efficacy of carnitine (Car) has also been started to be investigated [14–16].

Carnitine plays an important physiological role in the transport of long-chain fatty acids through the mitochondrial membrane for their beta-oxidation, and in ATP production in peripheral tissues [17]. Furthermore, it has been indicated that free carnitine and acylcarnitine strengthen the antioxidative defence mechanism of the

organism by forming chelates with metal ions that are involved in the initiation and continuation of oxidative reactions [18] and by scavenging and eliminating superoxide and hydrogen peroxide [14, 19].

This experiment was conducted to evaluate the effects of L-carnitine on performance, egg quality and serum glucose, protein, lipid, enzyme, mineral levels, liver and kidney histopathology and plasma malondialdehyde and nitric oxide levels in laying hens fed a diet containing high levels of copper proteinate (CuP).

Materials and Method

Forty-eight 42-week-old *Bovans* laying hens were randomly assigned to four treatment groups, four replicates of three animals each. The laying hens were fed with a basal diet

Table 1 Ingredients and chemical composition of basal diet fed to laying hen

Components of basal diet (%)	
Ingredients	
Corn	35.00
Barley	15.00
Sunflower meal (with 32% crude protein)	20.00
Fullfat soy bean	7.00
Soy bean meal (with 44% crude protein)	6.00
Wheat middling	3.80
Vegetable oil	3.50
Limestone	8.60
Methionine	0.05
Dicalcium phosphate	0.50
Lysine	0.10
Salt	0.25
Vitamin–mineral mixture ^a	0.20
Calculated values	
Metabolizable energy (kcal/kg)	2,650
Crude protein	17.0
Crude fat	6.0
Crude cellulose	7.0
Calcium	3.5
Phosphorus	0.7
Chemical analysis	
Dry matter	91.2
Crude protein	17.1
Crude fat	5.8
Crude cellulose	6.1
Crude ash	11.6

^a Provided per kilogramme of diet: vitamin A 6,400 IU, vitamin D₃ 1,600 IU, vitamin E 16 mg, vitamin K₃ 2.4 mg, vitamin B₁ 1.2 mg, vitamin B₂ 3.2 mg, nicotinamide 6.4 mg, calcium-D-panthotenate 4.8 mg, vitamin B₆ 2 mg, vitamin B₁₂ 0.012 mg, folic acid 0.4 mg, D-biotin 0.08 mg, vitamin C 16 mg, choline chloride 160 mg, Ca 444.24 mg, Mn 64 mg, Fe 24 mg, Zn 48 mg, Cu 4 mg, Co 0.16 mg, I 0.8 mg, Se 0.12 mg

(control) or the basal diet supplemented with either 400 mg Car (Carniking®, Vimar, Istanbul/Turkey)/kg diet, 800 mg CuP (Bioplex Copper, Alltech, Nicholasville, KY/USA)/kg diet or 400 mg carnitine + 800 mg copper (Car+CuP)/kg diet, for a period of 6 weeks.

Ingredients and chemical composition of basal diet are shown in Table 1. All animals were exposed to a 17-h light/7-h dark schedule and were housed in a pen within a temperature range of 18–20°C. The animals were given ad libitum feed and water.

The laying hens were weighed at the beginning and end of the study, and their live weights were recorded. Egg production was recorded on a daily basis. Feed consumption and feed efficiency were determined at 2-week intervals. The feed efficiency was calculated as the amount of feed consumed for the production of 1 kg of eggs.

Forty eggs (ten eggs from each replicates) were collected at three weekly intervals to determine egg specific gravity and egg shell thickness. Egg shell thickness was measured by micrometre (Mitutoyo, 0.01 mm, Japan). Specific gravity of a whole egg (gram per cubic centimetre) was measured by the Archimedes's method [20, 21].

At the end of the study, blood samples were collected from nine animals in each group by puncturing the brachial vein into dry and heparin-coated tubes. The blood samples were centrifuged at 3,000 rpm at room temperature for 10 min with an aim to extract serum and plasma. In serum samples, alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), and lipase activities, and triglyceride, total cholesterol, total protein, glucose, calcium (Ca), inorganic phosphor (Pi) and magnesium (Mg) concentrations were measured with an auto-analyser (Beckman LX-20 Coulter, Ireland) using a commercial kit (Beckman, Ireland). Plasma malondialdehyde (MDA) analyses were performed in accordance with the method described by Yoshioka et al. [22] based on the measurement of absorbance of the coloured complex resulting from the reaction of MDA with thiobarbituric acid. This coloured complex was extracted with *n*-butanol and measured at 532 nm spectrophotometrically. Plasma nitric oxide (NO) analyses were determined based on the measurement of absorbance of the complex formed by the reaction of nitrite with the Griess reaction, at 540 nm, as described by Aydin et al. [23]. MDA and NO analyses were performed spectrophotometrically (Schimadzu UV-1700), and the results were given as micromoles per litre.

At the end of the study, nine hens from each group were euthanized by cervical dislocation, and their liver and kidneys were extracted. The organs were weighed, and the results were recorded. Liver and kidney weight ratios were determined by calculating the weight of the liver and kidneys per 100 g of live weight.

Some parts of the liver samples were preserved in buffered formalin for histopathological processing. Tissue samples were embedded in paraffin, sectioned in 5–6 µm, stained with hematoxylin–eosin (H&E) and examined under light microscope [24].

The data obtained were analysed statistically using the SPSS 13.0 software. The significance of the differences between the groups was determined with one-way analysis of variance. Differences between groups were detected by Duncan's multiple range test. Data were given as mean±standard error.

Results

In the present study, the live weight, liver weight ratio, kidney weight ratio and egg weight were determined not to differ significantly in the all treatment groups compared to the control group

Table 2 Effects of dietary carnitine and copper supplementation on live weight, liver and kidney weight ratio in laying hens

Parameters	Control	Car	CuP	Car+CuP	<i>P</i> value
	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	
Initial live weight, g	1,505.05±31.80	1,518.00±41.66	1,502.00±41.20	1,516.17±58.41	<i>p</i> >0.05
Final live weight, g	1,457.42±21.22	1,428.67±37.34	1,391.42±40.02	1,405.08±45.18	<i>p</i> >0.05
Liver weight ratio, %	2.11±0.07	1.91±0.12	2.09±0.07	1.90±0.10	<i>p</i> >0.05
Kidney weight ratio, %	0.73±0.04	0.97±0.14	0.81±0.02	0.80±0.03	<i>p</i> >0.05

n=12

Car basal diet supplemented with 400 ppm L-carnitine, *CuP* basal diet supplemented with 800 ppm copper proteinate, *Car+CuP* basal diet supplemented with 400 ppm L-carnitine and 800 ppm copper proteinate

(*p*>0.05; Tables 2 and 3). The feed consumption (*p*<0.01), egg production and feed efficiency (*p*<0.001) were significantly lower in the CuP group when compared with the control.

The egg productions and feed efficiency were significantly higher (*p*<0.01) in the Car+CuP group than in the CuP group (Table 3). Furthermore, in the study, egg specific gravity was not altered (*p*>0.05), egg shell thickness had increased (*p*<0.001) in the Car group compared to the control group, and that both parameters had decreased in the CuP and the Car+CuP groups (*p*<0.001).

Except for a decrease in serum LDH activity, serum parameters were not altered significantly in the Car group. Serum ALT (*p*<0.05) and ALP (*p*<0.01) activities were determined to have increased in the CuP and the Car+CuP groups (Table 4). Serum LDH activity had decreased significantly in the CuP and the Car+CuP groups (*p*<0.001). On the other hand, AST, GGT and lipase activities and total cholesterol, total protein, triglyceride, Ca, P and Mg levels were determined not to have altered (*p*>0.05; Tables 4 and 5). Serum glucose levels had increased in the Car+CuP group compared to the control group (*p*<0.05; Table 5). The present study demonstrated that plasma MDA (*p*<0.01) and NO (*p*≤0.05) levels increased in the CuP group compared to the control. The level of MDA and NO was

Table 3 Effects of dietary carnitine and copper supplementation on performance, egg weight, egg shell thickness and egg specific gravity in laying hens

Parameters	Control	Car	CuP	Car+CuP	<i>P</i> value
	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	
Egg production, %	85.38±0.39a	83.55±1.39a	41.95±4.63c	56.56±3.74b	<i>p</i> <0.001
Feed consumption, g/day/hen	104.72±2.85a	99.04±2.50a	79.01±7.32b	81.33±2.75b	<i>p</i> <0.01
Feed efficiency (kg feed/kg egg)	2.03±0.05c	1.97±0.06c	3.19±0.10a	2.42±0.09b	<i>p</i> <0.001
Egg weight, g	60.37±0.36	60.25±0.56	59.41±0.81	59.68±0.31	<i>p</i> >0.05
Egg shell thickness, mm×10 ⁻²	33.20±0.005b	34.91±0.004a	31.83±0.005c	31.53±0.005c	<i>p</i> <0.001
Egg specific gravity, g/cm ³	1.0855±0.0014a	1.0851±0.0011a	1.0790±0.0015b	1.0812±0.0013b	<i>p</i> <0.001

The values within the same row with different lowercase letters differ significantly

Car basal diet supplemented with 400 ppm L-carnitine, *CuP* basal diet supplemented with 800 ppm copper proteinate, *Car+CuP* basal diet supplemented with 400 ppm L-carnitine and 800 ppm copper proteinate

Table 4 Effects of dietary carnitine and copper supplementation on some serum enzyme activities in laying hens

Parameters	Control	Car	CuP	Car+CuP	<i>P</i> value
	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	
AST, IU/L	171.11±5.66	186.22±7.20	195.25±10.20	190.50±12.53	<i>p</i> >0.05
ALT, IU/L	6.90±0.38b	8.52±0.38ab	9.44±0.96a	9.29±0.75a	<i>p</i> <0.05
GGT, IU/L	37.45±1.38	37.10±1.79	34.00±1.61	33.57±1.48	<i>p</i> >0.05
ALP, IU/L	406.64±49.67b	342.60±37.10b	646.70±118.83a	703.71±90.11a	<i>p</i> <0.01
LDH, IU/L	749.91±43.21a	380.50±38.73b	400.30±39.62b	420.71±50.93b	<i>p</i> <0.001
Lipase, IU/L	17.09±1.47	17.40±1.73	14.90±0.71	16.00±1.85	<i>p</i> >0.05

n=9; the values within the same row with different lowercase letters differ significantly

Car basal diet supplemented with 400 ppm L-carnitine, *CuP* basal diet supplemented with 800 ppm copper proteinate, *Car+CuP* basal diet supplemented with 400 ppm L-carnitine and 800 ppm copper proteinate

found to be reduced by the *Car+CuP* supplementation as compared with the group treated with *CuP* alone. On the other hand, in the *Car* group, no significant alteration was observed, when compared to the controls (Table 5).

The histopathological examination of the liver of hens receiving *CuP* in diet revealed hepatocyte degeneration of different types (parenchymal and vacuolar degenerations). The remark cords were dissociated. Parenchymal and vacuolar degenerations were marked in hepatocytes (Fig. 1c). Microscopic examinations showed that the hepatic lesions induced by *CuP* supplementation were remarkably reduced by the combination carnitine and copper. (Fig. 1d). No significant lesion was determined in the kidney of hens fed a diet supplemented with either *CuP* or *Car* (Fig. 2b, c).

Table 5 Effects of dietary carnitine and copper supplementation on serum glucose, lipid, protein, mineral and plasma MDA and NO levels in laying hens

Parameters	Control	Car	CuP	Car+CuP	<i>P</i> value
	Mean±SEM	Mean±SEM	Mean± SEM	Mean±SEM	
Total cholesterol, mg/dl	116.82±11.22	89.90±9.67	128.80±19.06	130.29±18.03	<i>p</i> >0.05
Triglyceride, mg/dl	1,082.36±152.02	701.80±136.80	636.50±189.58	644.00±260.37	<i>p</i> >0.05
Total protein, mg/dl	4.73±0.47	4.20±0.25	4.70±0.21	4.00±0.22	<i>p</i> >0.05
Glucose, mg/dl	221.36±2.34b	219.70±6.11b	234.40±5.76ab	238.86±6.85a	<i>p</i> <0.05
Ca, mg/dl	20.43±1.57	17.97±0.99	18.79±2.17	14.45±1.77	<i>p</i> >0.05
Pi, mg/dl	4.39±0.25	4.49±0.44	4.20±0.40	3.46±0.34	<i>p</i> >0.05
Mg, mg/dl	1.31±0.03	1.22±0.05	1.24±0.07	1.14±0.11	<i>p</i> >0.05
MDA, μmol/L	5.51±0.71b	7.35±0.64b	10.33±1.35a	6.98±0.73b	<i>p</i> <0.01
NO, μmol/L	20.14±2.3b	16.48±1.7b	32.21±1.9a	21.86±2.1b	<i>p</i> ≤0.05

n=9; the values within the same row with different lowercase letters differ significantly

Car basal diet supplemented with 400 ppm L-carnitine, *CuP* basal diet supplemented with 800 ppm copper proteinate, *Car+CuP* basal diet supplemented with 400 ppm L-carnitine and 800 ppm copper proteinate

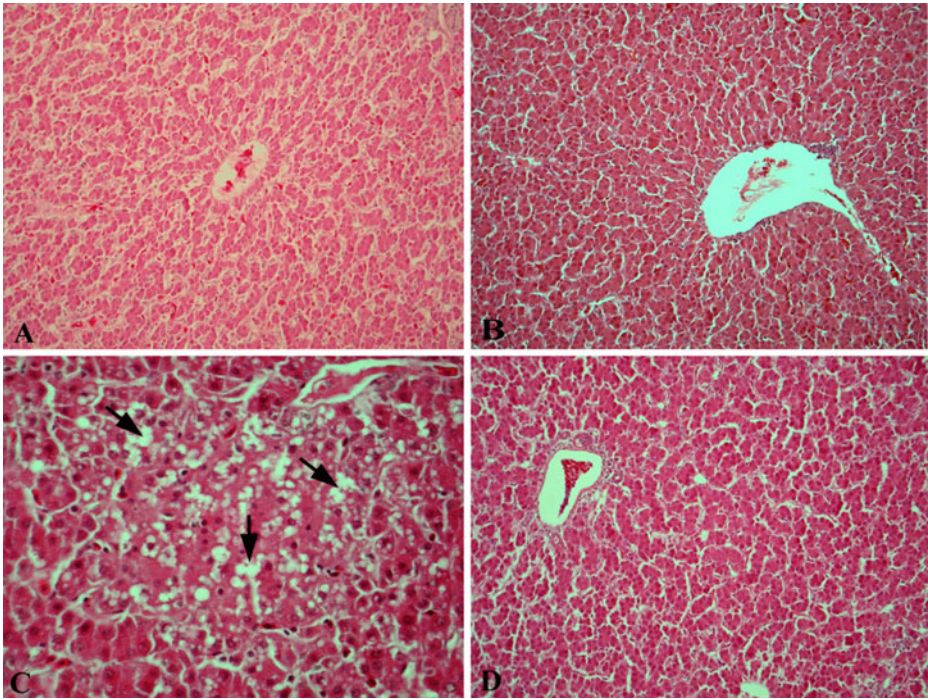


Fig. 1 **a** The appearance of normal liver tissue (control group, H&E, $\times 200$). **b** No significant lesion was determined in the liver cross sections (Car group H&E, $\times 200$). **c** Parenchymal and vacuolar degenerations (*arrows*) were marked in hepatocytes (CuP, H&E, $\times 400$). **d** No significant lesion was determined in the liver cross sections (Car+CuP group, H&E, $\times 200$)

Discussion

It is reported that the effect of copper on the performance of hens is closely related to the dose supplemented. The addition of 100–250 mg/kg of organic or inorganic Cu to poultry feed affects performance positively [4–6]. Long-term exposure to high levels (500–3,000 ppm) of Cu with or without the association of several environmental factors may cause reduced performance, retarded growth, epithelial erosion in the digestive tract, hepatic necrosis, obstruction of the biliary ducts and icterus as well as the peroxidation of membrane lipids [1, 4, 25–27]. Similarly, the observation of reduced feed consumption [10, 25, 26, 28], feed efficiency [10] and egg production [8, 25] in the CuP group in the present study was in accordance with the above-mentioned reports. In CuP group, feed consumption decreased significantly, but live weight was not decreased. This is related to the short duration time of research. The adverse effect of high levels of Cu on the performance of animals could be related to either the sloughing of epithelial cells in the digestive tract [1], reduced feed consumption and resulting nutrient deficiencies [29], or to the oxidative effect of Cu [1, 25]. On the other hand, the statistically significant increase observed in egg productions and feed efficiency and the slight increase in feed consumption (which was reduced upon the addition of high copper proteinate to diet) by the addition of an equal dose of Car with CuP suggest that Car could induce a positive effect in CuP intoxication cases.

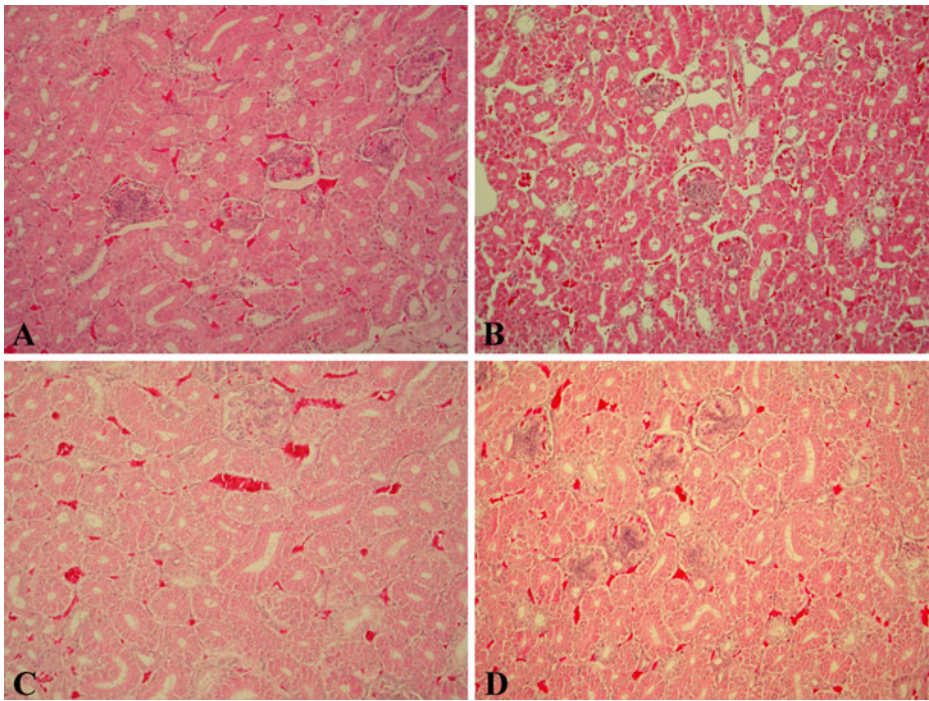


Fig. 2 a Normal appearance of the kidneys. Control group, H&E, $\times 200$; b Car group H&E, $\times 200$. No significant lesion was determined in the kidney cross sections, c CuP, H&E, $\times 400$; d Car+CuP group, H&E, $\times 200$

In accordance with the results of the present study, Tekeli et al. [30] reported that the addition of 500 ppm of copper sulphate to laying hen diets reduced egg shell thickness. The significant decrease observed in egg shell thickness and egg specific gravity in the CuP and the Car+CuP groups compared to control demonstrated that supplementation of L-carnitine did not alleviate or prevent the adverse effects of copper proteinate on these parameters. No significant alteration in AST, ALP, ALT, GGT or lipase activities, excluding LDH, in the Car groups is in agreement with Guclu et al. [31].

When taken into the body, Cu is stored in the liver after binding to albumin. Therefore, in the case of the intake of high levels of Cu, the first organ which is adversely affected is the liver [1]. On the other hand, in the event of a disease or intoxication affecting the liver, the cell membrane of hepatocytes is damaged, which results in the rapid increase of the plasma levels of amino transferases. Therefore, these enzymes are accepted to be major indicators of liver damage [32]. Similar to literature reports [4], it was determined in the present study that in the CuP group, serum ALP and ALT activities had increased significantly compared to the control. Furthermore, the increased serum ALP and ALT activities determined in this group were inconsistent with the degenerative alterations determined in the histopathological examination of the liver (parenchymal and vacuolar degeneration). Although it is reported that increased ALP activity in avian species should be interpreted as an indicator of hepatic and renal dysfunction [4], in the present study, no significant alteration was determined in the histopathological examination of the kidneys. Similar to the report of Mansour [15] indicating that acetyl-L-carnitine administration did not have any significant effect on increased serum AST, ALT and GGT activities, which had

been induced by oxidative stress, the present study did not reveal any significant alteration in the activities of these enzymes in the Car+CuP group. Consistent with the results of the study conducted by Guclu et al. [8], the present study demonstrated that the administration of 800 ppm of copper proteinate also reduced serum LDH activity.

Carnitine plays an important role in the transfer of long-chain fatty acids to the mitochondrial matrix for oxidation, facilitates the β -oxidation of fatty acids, thereby decreasing serum total cholesterol and triglyceride levels and inducing a hypolipidemic effect [33]. Although a slight reduction was determined in total cholesterol and triglyceride levels in the group receiving 400 mg/kg Car in diet, these decreases were found not to be significant statistically compared to control. Similarly, Guclu et al. [31] indicated that 200 ppm of L-carnitine did not affect serum cholesterol or triglyceride levels. Some studies have reported that levels of Cu increased significantly serum glucose in chicken [4, 8]. In the present study, although a slight increase was observed in serum glucose levels in the CuP group, a statistically significant increase was observed only in the Car+CuP group.

Lipid peroxide radicals, which are generated as a result of lipid peroxidation caused by reactive oxygen species, can be converted into highly cytotoxic products such as MDA [34]. Lipid peroxide radicals cause cellular damage by either increasing the permeability of the cell membrane or directly binding to the DNA or other macromolecules of the cell, such as proteins [1]. Previous studies have shown that high levels of Cu induced lipid peroxidation in rats, thereby increasing serum and brain MDA levels [35, 36]. The occurrence of oxidation and reduction reactions between copper ions (Cu^{+1} , Cu^{+2}) and reactive oxygen species, generated as a result of disorders in the biochemical reactions of the organism, brings about the reaction of cuprous copper (Cu^{+2}) with superoxide anion radicals or other agents (i.e. ascorbic acid, glutathione) and its reduction to cupric copper (Cu^{+1}), and the oxidation of Cu^{+1} with hydrogen peroxide (H_2O_2), generating Cu^{+2} once again and hydroxyl radicals, which have very strong oxidative effect (Haber–Weiss reaction). This mechanism is considered to induce lipid peroxidation in the organism [1].

In the present study, the significant increase observed in plasma MDA and NO levels in the CuP group demonstrated that high levels of Cu cause peroxidative damage to membrane lipids. This finding is supported by the results of previous studies [8, 37].

Mansour [15] reported that the administration of acetyl-L-carnitine to rats suffering from gamma irradiation-induced lipid peroxidation decreased tissue MDA and NO levels. In the present study, plasma MDA and NO levels having decreased significantly in the CuP+Car group supports the report that carnitine exhibits antioxidative effect [14–16]. The antioxidant effect of carnitine could be attributed to its maintaining membrane stabilization, thus, protecting the cell and mitochondrion from damage [38], and reducing the transport of free radicals through the membrane, as well as its scavenging the hydroxyl [19], anion radicals, and H_2O_2 , generated as a result of the Fenton reaction [14], thus limiting their adverse effects.

Conclusion

In conclusion, in the present study, it was determined that high levels of copper proteinate added to laying hen diets caused adverse effects on performance, egg quality and some enzyme parameters, and aggravated lipid peroxidation, whereas the addition of L-carnitine to feed containing high levels of copper proteinate produced a positive effect on performance and lipid peroxidation.

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