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Influences of the Dietary Supplementation of Broiler Chickens with Copper Nanoparticles on Some Biometric Measurements and Transcription of Copper-allied Genes

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Abstract

This study was conducted to investigate the effects of the sources and levels of dietary copper supplementation on broiler chickens. A 37-day feeding trial was accomplished using 192 one-day-old, unsexed Arbor Acres broiler chicks which were randomly allocated into four groups with different strategies of copper supplementation. The first group was the negative control and fed on diets containing copper-free mineral-vitamin broiler premix. Diets of the second group were supplemented with copper sulfate (Cu_2SO_4) at the level of 7.5 mg/kg. The third and fourth groups were fed on diets supplemented with copper nanoparticles (Cu-NPs) at the levels of 7.5 mg/kg and 3.75 mg/kg, respectively. Growth performance, carcass characteristics, copper concentrations, serum antioxidant biomarkers and mRNA of metallothionein and ceruloplasmin were evaluated. Results of growth performance showed that Cu-NPs significantly ($P \leq 0.05$) increased body weight gain and feed utilization compared to an equal quantity of Cu_2SO_4. No adverse effect on growth performance occurred when Cu-NPs were supplemented at half of the level of Cu_2SO_4. Dressing and thigh yields were significantly ($P \leq 0.05$) increased in birds in the fourth group. Total antioxidant capacity, malondialdehyde level and transcription of ceruloplasmin were not significantly affected by the tested copper sources or levels. The superoxide dismutase enzyme activity and transcription of metallothionein were significantly ($P \leq 0.05$) increased by the replacement of Cu_2SO_4 with the same quantity of Cu-NPs. The obtained findings suggested that Cu-NPs could be a superior dietary supplement of copper in broiler chickens' diets over Cu_2SO_4, with possibility of quantity reduction.

KEYWORDS
Broilers, Carcass characteristics, Ceruloplasmin, Copper nanoparticles, Metallothionein

INTRODUCTION

Copper (Cu) is an essential trace element involved in several physiological and biochemical processes in poultry (Wen *et al*., 2019). It is a component of the integral active parts of numerous metalloenzymes such as superoxide dismutases, cytochrome-c oxidase, hephaestin, lysyl oxidases and others (Suttle, 2010; Hefnawy and Elkhaiat, 2015). The dietary level of Cu interferes with the genetic expression, thereby the function, of some proteins including metallothionein and ceruloplasmin (González *et al*., 2008; Song *et al*., 2009). The variable content and bioavailability of Cu in feedstuffs make it a prevalent practice to regularly supplement diets with this element (Leeson, 2009). The poultry industry relies on Cu sulfate (Cu_2SO_4) as a Cu supplement owing to the economic rationality. However, several problems are ascribed to the physicochemical nature of Cu_2SO_4 which downgrades the nutritive value of expensive feed components such as phosphorous, phytase enzyme (Banks *et al*., 2004), and vitamins (Marchetti *et al*., 2000; Luo *et al*., 2005). Besides, Cu_2SO_4 is poorly absorbed and highly excreted causing serious environmental hazards (Scott *et al*., 2017a). Organic Cu sources, such as Cu-glycine and Cu-methionine, have shown a better utilization efficiency than the inorganic Cu_2SO_4 (Świątkiewicz *et al*., 2014; Kwiecien *et al*., 2015). Nevertheless, organic sources are not widely applied in broiler chicken nutrition possibly due to the higher cost relative to Cu_2SO_4 or the difficulty in evaluation of bioavailability (Bao and Choct, 2009). The special properties of nanoparticles made the nano-size Cu become a promising supplement of Cu due to the high utilization and cost-efficacy of Cu nanoparticles (Sharif *et al*., 2021). The increased bioavailability of such sources has been attributed to the small particle size and the large active surface area of Cu nanoparticles (Cu-NPs), with a potentiality of quantity reduction (Sawosz *et al*., 2018; El-Kassas *et al*., 2019). Numerous studies have compared the influences of Cu_2SO_4 and Cu-NPs on broiler chickens' performance, however, the findings were uncertain, especially in terms of the growth performance and homeostasis of Cu inside the body (Scott *et al*., 2017a). Some authors have reported an improvement in the growth performance as a result of the supplementation of Cu-NPs to broiler chickens via different methods of administration (Wang *et al*., 2011; Miroshnikov *et al*., 2015; Mroczek-sosnowska *et al*., 2015; Joshua *et al*., 2016; Scott *et al*., 2017b; Kazaz and Hafez, 2020; Yausheva, 2021). Whereas others have reported that there were no significant impacts on the growth performance of broilers by using Cu-NPs (Ognik *et al*., 2017; Kozłowski *et al*., 2018; Lee *et al*., 2021; Kim *et al*., 2022). Additionally, the recommended synthesis tech-

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niques of Cu-NPs and inclusion levels need further confirmation. Hence, the current study aimed to explore the outcomes of the dietary supplementation of broiler chickens with Cu-NPs on the growth performance, carcass characteristics, Cu concentrations, serum antioxidant biomarkers and hepatic transcription of metallothionein (MT) and ceruloplasmin (CP).

MATERIALS AND METHODS

This protocol was approved by the Institutional Animal Care and Use Committee, Faculty of Veterinary Medicine, Cairo University, Egypt (vet CU-2303-2022445). The broiler chickens were housed at the Animal and Poultry Research Center, Faculty of Veterinary Medicine, Cairo University, Egypt.

Synthesis and characterization of copper nanoparticles

Cu-NPs were synthesized through a chemical reduction technique (Khan *et al*., 2015), where $(C_6H_{10}O_5)n$ and $CuSO_4.5H_2O$ (Sigma-Aldrich) were used as reactants. X-ray diffractometer (X'Pert Pro, PANalytical), high-resolution transmission electron microscopy (JEM-2100F 200 KV, JEOL) and dynamic light scattering analyzer (Zetasizer Nano-ZS90 instrument, Malvern) were utilized for determination of crystallography, morphology, distribution, and particle size of the prepared nanoparticles.

Animals' husbandry and experimental design

A total of 192 one-day-old, unsexed Arbor Acres broiler chicks with an average body weight of 39.1±0.8 g were randomly distributed among four groups. Each group was divided into 4 replicates (12 chicks per replicate). All birds were housed in experimental floor pens provided with saw-dust bedding. The temperature of the pens was adjusted to 32°C during the first week then has been gradually lowered by 2°C/week, and a continuous lighting system was applied. The birds have had free access to tap water and feed. Birds were fed the same basal diet in a processed form for 37 days divided into 3 phases (starter 0 to 10 d, grower 11 to 24 d, and finisher 25 to 37 d). The diets (Table 1) were formulated according to the nutrient specifications for Arbor Acres broiler chickens (Aviagen, 2019). The groups were designed according to the sources and/or the levels of the Cu supplements in the broiler mineral-vitamin premix. Where the first group (G1) was supplied with Cu-free premix (negative control), the second group (G2) was supplied with a premix that contains Cu_2SO_4 at the level of 2500 mg/kg, while the third and fourth groups (G3 and G4) were supplied with broiler premixes that contain 2500 mg/kg and 1250 mg/kg of Cu-NPs, respectively. Premix was added at the level of 3 kg/ton of diet (Table 2). The analyzed Cu content in feedstuffs (without a Cu supplement) was 3.83 mg/kg of diet.

Growth performance evaluation

The initial body weight of the chicks was measured on arrival. Feed intake (FI), body weight (BW) and body weight gain (BWG) of birds were recorded in each replicate on the 10th, 25th and 37th days of age. Feed conversion ratio (FCR) was calculated as FCR =

Table 1. Ingredients and calculated nutrient composition of the experimental diets.

	Starter (Days 0-10)	Grower (Days 11-24)	Finisher (Days 25-37)
Ingredients (%)			
Yellow corn	54.4	57.1	59.8
Corn gluten meal, 62% crude protein	4.3	3.55	3.4
Soybean meal, 47% crude protein	34.4	31.7	28.3
Soybean oil	2.5	3.7	4.8
Mono-calcium phosphate	1.5	1.35	1.21
Limestone, ground	1.55	1.4	1.32
Common salt (NaCl)	0.4	0.4	0.4
Broiler chickens mineral–vitamin premix[a, b]	0.3	0.3	0.3
DL-Methionine, 99%	0.31	0.27	0.26
L-Lysine, 98%	0.19	0.13	0.12
L-Threonine, 98.5%	0.15	0.1	0.09
Total	100	100	100
Calculated nutrient composition (As fed)			
Crude protein (%)	23.03	21.5	20.02
Calcium (%)	0.96	0.87	0.8
Available phosphorus (%)	0.48	0.44	0.41
Lysine (%)	1.44	1.29	1.19
Methionine (%)	0.71	0.65	0.62
Methionine + Cystine (%)	1.08	0.99	0.95
Threonine (%)	0.98	0.88	0.81
Metabolizable energy (kcal/kg)	3000.2	3101.9	3200.9

[a]Each kilogram contains 1200000 IU Vit. A, 350000 IU Vit. D3, 4000 mg Vit. E, 250 mg Vit. B1, 800 mg Vit. B2, 600 mg Vit. B6, 3.2 mg Vit. B12, 450 mg Vit. K3, 4.5 g nicotinic acid, 1.5 g calcium pantothenate, 120 mg folic acid, 5 mg biotin, 55 mg choline chloride, 3 g iron sulfate, 10 g manganese oxide, 8 g zinc oxide, 0.15 mg sodium selenite, 120 mg sodium iodide and 40 mg cobalt sulfate.
[b]Copper source and level in the premix depend on the group of birds (see Table 2).

feed intake (g) / body weight gain (g).

Carcass characterization and samples collection

At the 37th day of age, and after two hours of feed withdrawal, five birds per replicate were randomly picked, then slaughtered by jugular vein severing. The blood samples were collected in separate vacuum tubes and the centrifuge at the speed of 1,000 g was used for 15 min at 4°C to obtain the blood serum. The collected serum samples were immediately frozen at −20°C for further analyses. After complete evisceration, the dressing yield and the indices of liver and spleen were calculated (as a percent of live body weight). The breast and thigh yields were measured (as a percent of carcass weight). Liver and breast muscle tissues were sampled, then immediately frozen at −80°C in liquid nitrogen for further analyses.

Serum antioxidant biomarkers

All samples were colorimetrically analyzed using a UV/visible double beam spectrophotometer (Model: 6850, Jenway). The total antioxidant capacity (TAC) was determined as indicated by Koracevic *et al*. (2001). The activity of the superoxide dismutase enzyme (SOD) was measured according to the procedures of Nishikimi *et al*. (1972). The level of malondialdehyde (MDA) was analyzed as described by Mitsuru and Midori (1978).

Copper concentrations analyses

The collected serum, breast muscle and liver samples were subjected to a preparation process followed by the determination of Cu concentrations using an atomic absorption spectrometer (iCE 3300, Thermo Scientific) as detailed by Kurnaz and Filazi (2011).

Genomic assay

Total RNA was extracted from 30 mg of frozen liver tissue (Chomczynski and Sacchi, 1987) using RNA purification kits (GeneJET K0731, Thermo Fisher Scientific Inc.). First-strand cDNA synthesis kits (RevertAid K1621, Thermo Fisher Scientific Inc.) were utilized to obtain first-strand cDNA which has been exposed to PCR amplification (Wiame *et al*., 2000) by DNA polymerase enzyme (DreamTaq, Thermo Scientific). The measurement of mRNA levels was performed on a Real-Time PCR System (CFX96, BioRad) with a 20 µl reaction. Primer sequences of MT, CP and beta-actin (ACTB) are presented in Table 3. Fold changes were expressed as $2^{-\Delta\Delta Ct}$. The mRNA levels were normalized to the mean transcription of ACTB as a reference gene.

Statistical analyses

The data were presented as mean ± standard error (SE). All data were analyzed by ANOVA test with LSD multiple comparison post hoc, using an SPSS statistical software package (version 26.0; SPSS Inc.). The probability value (P value) of ≤ 0.05 was defined to indicate statistical significance.

RESULTS

Characterization of the prepared nanoparticles showed that the synthesized Cu-NPs have a uniform spherical shape with an average particle size of 72 nm (Figure 1). Results of the growth performance and carcass characterization of the broiler chickens at the age of 37 days are summarized in Table 4. The feed intake of all birds was not significantly influenced. While the Cu-NPs supplementation at the level of 7.5 mg/kg of diet significantly (P ≤ 0.05) improved the BW, BWG and FCR compared to the other groups. The dressing and thigh yields were significantly (P ≤ 0.05) higher in birds in G4 than in other birds. Also, birds in G1 had a higher liver index compared to other groups. Data of serum antioxidant biomarkers and Cu concentrations analyses are presented in Table 5. Cu supplementation did not induce a significant change in TAC and MDA levels in the blood serum of all birds. However, the activity of the SOD enzyme was significantly (P ≤ 0.05) increased in broiler chickens supplemented with Cu compared to unsupplemented birds. The broilers provided with Cu-NPs-containing diets (G3 and G4) achieved significantly (P ≤

Table 2. Copper sources and levels in the experimental diets of broiler chickens

Item	G1 (Negative control)[a]	G2	G3	G4
Copper sulfate (mg/kg of premix)	0	2500	0	0
Copper sulfate (mg/kg of diet[b])	0	7.5	0	0
Copper nanoparticles (mg/kg of premix)	0	0	2500	1250
Copper nanoparticles (mg/kg of diet[b])	0	0	7.5	3.75

[a]Basal diet without copper supplementation.
[b]Premix was added at the level of 3 kg/ton of diet.

Table 3. Accession number and mRNA primer sequences of the measured genes[a]

Item	Accession number		Primer sequence (5'-3')
Gallus gallus ceruloplasmin (CP)	XM_040705988.2	Forward	TACCACAAGAGCAACGAGGG
		Reverse	AGAGCTCCTTTCCTGCACAC
Chicken metallothionein (MT)	X06749.1	Forward	ACCCGAACTGAACCATGGAC
		Reverse	TTTTCGTGGTCCCTGTCACC
Gallus gallus actin, beta (ACTB)[b]	NM_205518.2	Forward	ATGAAGCCCAGAGCAAAAGA
		Reverse	GGGGTGTTGAAGGTCTCAAA

[a]Designed by NLM: NCBI (National Library of Medicine: The National Center for Biotechnology Information https://www.ncbi.nlm.nih.gov, accessed 17 January 2022).
[b]Reference gene.

Table 4. Growth performance, feed utilization and carcass characteristics of broiler chickens fed on diets supplemented with different sources and levels of copper

Item	G1 (Negative control)[a]	G2 (Cu_2SO_4[b], 7.5 mg/kg)	G3 (Cu-NPs[c], 7.5 mg/kg)	G4 (Cu-NPs[c], 3.75 mg/kg)	P value
Growth performance and feed utilization					
Feed intake (FI, g)	3666.83 ± 14.91	3680.96 ± 18.34	3494.09 ± 116.12	3607.96 ± 16.76	0.185
Body weight (g)	2205.00 ± 12.06[b]	2246.33 ± 33.58[b]	2331.00 ± 23.07[a]	2248.50 ± 19.34[b]	0.019
Body weight gain (BWG, g)	2166.75 ± 12.72[b]	2207.08 ± 33.63[b]	2292.25 ± 23.46[a]	2208.50 ± 19.92[b]	0.021
Feed conversion ratio (FI / BWG)	1.694 ± 0.005[a]	1.669 ± 0.028[a]	1.526 ± 0.038[b]	1.634 ± 0.007[a]	0.009
Carcass characteristics					
Dressing yield (% of live body weight)	71.78 ± 0.33[b]	72.20 ± 0.33[b]	71.91 ± 0.67[b]	74.26 ± 0.50[a]	0.002
Breast yield (% of carcass weight)	28.32 ± 0.60	27.52 ± 0.59	27.29 ± 1.0	27.34 ± 0.78	0.756
Thigh yield (% of carcass weight)	41.11 ± 0.56[b]	41.09 ± 0.46[b]	41.14 ± 0.53[b]	44.48 ± 0.70[a]	0.000
Liver index (% of live body weight)	3.61 ± 0.14[a]	2.90 ± 0.14[b]	2.88 ± 0.13[b]	2.74 ± 0.21[b]	0.002
Spleen index (% of live body weight)	0.18 ± 0.02	0.17 ± 0.01	0.18 ± 0.01	0.15 ± 0.02	0.706

[a]Basal diet without copper supplementation. [b]Copper sulfate. c Copper nanoparticles.
Data are presented as Mean ± SE (Standard error).
Values in the same row with different superscripts are significantly different according to ANOVA (LSD, P ≤ 0.05).

0.05) higher SOD enzymatic activity than those supplemented with Cu_2SO_4 (G2). There was a significant (P ≤ 0.05) much Cu in the serum of birds which were received a Cu supplement in comparison with G1, where the significantly (P ≤ 0.05) highest Cu concentration in serum was detected in G3. The deposition of Cu in liver tissue exhibited significant (P ≤ 0.05) differences between all groups. The Cu content in breast muscle of birds in G3 was significantly higher than in other birds. Fold changes in the hepatic mRNA levels of MT and CP are illustrated in Figure 2. The transcription of MT in birds in G3 was significantly (P ≤ 0.05) upregulated relative to birds in other groups. The transcription of CP was unsensible for Cu supplementation under the study conditions.

Fig. 1. The Morphological characterization of the prepared copper nanoparticles was carried out using high-resolution transition electron microscopy imaging (200 kV, X30000). Images show that the prepared copper nanoparticles have a uniform spherical shape with an average particle size of 72 nm.

Fig. 2. Fold changes in the transcription of metallothionein (MT) and ceruloplasmin (CP) in the liver of broiler chickens fed on diets supplemented with different sources and levels of copper (Cu). The groups were G1 (Negative control, basal diet without Cu supplementation), G2 (Cu sulfate, 7.5 mg/kg of diet), G3 (Cu nanoparticles, 7.5 mg/kg of diet) and G4 (Cu nanoparticles, 3.75 mg/kg of diet). The mRNA levels of MT and CP were normalized to the mean transcription of beta-actin (ACTB) as a reference gene. The negative control group (G1) was taken as a benchmark to which other groups were compared and expressed as fold changes. G3 showed a significant (P ≤ 0.05) upregulation in MT transcription relative to other groups. CP transcription was not changed under the conditions of our study.

Table 5. Serum antioxidant biomarkers and copper concentrations in broiler chickens fed on diets supplemented with different sources and levels of copper.

Item	Group 1 (Negative control)[a]	Group 2 (Cu_2SO_4[b], 7.5 mg/kg)	Group 3 (Cu-NPs[c], 7.5 mg/kg)	Group 4 (Cu-NPs[c], 3.75 mg/kg)	P value
Serum antioxidant biomarkers					
Total antioxidant capacity (µmol/ml)	3.47 ± 0.330	3.85 ± 0.055	3.74 ± 0.110	3.63 ± 0.110	0.556
SOD[d] (µmol/ml)	90.79 ± 3.093[c]	105.65 ± 4.473[b]	117.35 ± 3.045[a]	115.98 ± 2.457[a]	< 0.0001
Malondialdehyde (nmol/ml)	3.06 ± 0.007	3.04 ± 0.007	2.90 ± 0.006	2.85 ± 0.005	0.228
Copper concentrations					
Serum (mg/L)	0.89 ± 0.041[c]	1.14 ± 0.036[b]	1.52 ± 0.042[a]	1.11 ± 0.040[b]	< 0.0001
Liver (mg/kg)	10.56 ± 0.42[d]	13.57 ± 0.42[c]	13.94 ± 0.71[a]	13.66 ± 0.82[b]	0.024
Breast Muscle (mg/kg)	2.28 ± 0.09[b]	2.43 ± 0.15[b]	2.95 ± 0.11[a]	2.53 ± 0.17[b]	0.001

[a]Basal diet without copper supplementation. b Copper sulfate. c Copper nanoparticles.
[d]Superoxide dismutase enzyme activity.
Data are presented as Mean ± SE (Standard error).
Values in the same row with different superscripts are significantly different according to ANOVA (LSD, P ≤ 0.05).

DISCUSSION

The results showed that the Cu-NPs supplementation of birds in G3 significantly achieved better growth performance, which was expressed as higher BW, BWG and reduced FCR, in comparison to other groups. These findings suggest that Cu-NPs could be provided to broiler chickens at half of the recommended level of the dietary Cu_2SO_4 supplementation without jeopardizing the growth performance of birds. Sawosz *et al*. (2018) have reported the positive effect of the dietary supplementation of Cu-NPs to broiler chicken on BWG and FCR over the use of Cu_2SO_4. Consistently, El-Kassas *et al*. (2019) have noticed that the dietary incorporation of Cu-NPs has ameliorated the inhibitory effect of chronic heat stress on BWG of broiler chickens. Likewise, Wang *et al*. (2011) have demonstrated the motivating effect of copper-loaded chitosan NPs on the growth performance of broiler chickens. In the same context, the *in ovo* injection of broiler chicken embryos with Cu-NPs has shown an improvement in the final body weight of the hatched chicks (Mroczek-sosnowska *et al*., 2015; Joshua *et al*., 2016; Scott *et al*., 2017b). Miroshnikov *et al*. (2015) and Yausheva (2021) have obtained resembling results after a single intramuscular injection of the broiler chickens with Cu-NPs-containing solutions. Similarly, the administration of Cu-NPs via drinking water to broiler chickens has induced a better growth performance vs. the administration of Cu_2SO_4 (Kazaz and Hafez, 2020). Parallel to our results, El-Kassas *et al*. (2019) have indicated the possibility of providing 50% of the recommended level of Cu_2SO_4 supplementation in the form of Cu-NPs without deteriorating the growth performance of broiler chickens. Whereas Sawosz *et al*. (2018) have suggested that the replacement of Cu_2SO_4 with Cu-NPs potentially reduces the used quantity by 75% without an adverse effect on the growth performance. Contrarily, Lee *et al*. (2021) and (Kim *et al*., 2022) have reported an absence of the influence of the dietary inclusion of graded levels (16 mg/kg, 40 mg/kg, 80 mg/kg, and 120 mg/kg) of the hot-melted extruded Cu sulfate (nano-size particles) on the growth performance of broiler chickens when compared to Cu_2SO_4. A similar conclusion has been indicated by Kozłowski *et al*. (2018) with broiler turkeys. Consistently, Ognik *et al*. (2017) have excluded the effect of the administration of Cu-NPs via drinking water on the growth of broiler chickens fed on a Cu-deficient diet (deficient by 29% of the recommended level of Cu requirements). The obtained results possibly are ascribed to the high utilization efficiency of Cu-NPs because of their different physicochemical and biological natures when compared to the micro-particles of Cu. The small particle size and the large surface area of Cu-NPs allow a prolonged residence time in the gastrointestinal tract and reduce the intestinal clearance mechanisms (Chen *et al*., 2006). Mainly by these two features, the diffusion of Cu-NPs through the intestinal barriers is more facilitated to be metabolized primarily by the liver and spleen (Singh, 2016). The absorbed Cu-NPs could also be distributed and retained in tissues irrespective of blood circulation (Anjum *et al*., 2016). As a result of the aforementioned pathways, Cu-NPs deliver more functional Cu to the cells relative to the micro-particles of Cu (Chen *et al*., 2006). The higher bioefficacy of Cu-NPs could improve the growth of birds by various mechanisms such as enhancement of iron absorption, hemoglobin synthesis and erythrocytes formation (Samanta *et al*., 2011). Cu-NPs may additionally increase the nutrient utilization and meat buildup of birds by upregulating the expression of growth hormone mRNA in the pituitary gland (LaBella *et al*., 1973), refining the regulatory peptides (Eipper and Mains, 1988), and favorably modifying the gut microflora due to the antimicrobial effect of Cu (Pang *et al*., 2009; Zheng *et al*., 2013). Cu-NPs can enhance the growth performance of birds by the incorporation into the active sites of dopamine-beta monooxygenase and tyrosine hydroxylase enzymes (Suttle, 2010). Moreover, Cu-NPs could promote growth by increasing the activity of lipase and phospholipase enzymes (Gonzales-Eguia *et al*., 2009; Das *et al*., 2009), and the utilization of amino acids that is partially indicated by the low levels of blood urea (Wang *et al*., 2011; Miroshnikov *et al*., 2015). Kazaz and Hafez (2020) have differently attributed the positive influence of Cu-NPs on BWG to the optimization of welfare-indicating behaviors of broiler chickens. The worse FCR of birds supplemented with Cu_2SO_4 compared to Cu-NPs at the same quantity might be a consequence of the adverse effects of Cu_2SO_4 on the utilization of valuable feed components such as some vitamins (Marchetti *et al*., 2000; Luo *et al*., 2005), phosphorous and phytase enzyme (Banks *et al*., 2004), in addition to its poor absorbability (Scott *et al*., 2017a). It is difficult to accurately explain the significant (P ≤ 0.05) increase in the dressing and thigh yields of birds supplemented with 3.75 mg/kg (G4) in comparison to birds fed on higher levels of Cu in the form of either Cu-NPs or Cu_2SO_4. However, slightly similar results were obtained with rats by Cholewińska *et al*. (2018), who have found that the animals that have received a low dose of Cu-NPs (3.25 mg/kg of diet) have exhibited an increased lean percent vs. those received a high dose (6.5 mg/kg of diet). This dose-dependent interaction could be ascribed to the accumulation of Cu-NPs inside the body at high levels of supplementation (A. Scott, personal communication on 18th, May 2022). Another possible reason for this interaction is the harmful effect of the high levels of Cu-NPs on the liver, spleen and kidneys (El Bialy *et al*., 2020). The higher liver index of G1 broilers than those in other groups probably be due to the relationship between Cu deficiency and fatty liver syndrome that has been studied in backyard chickens and humans (Trott *et al*., 2014; Ito, 2021). The dietary Cu supplementation has not led to any significant change in TAC and MDA levels among all birds. Whereas the activity of the SOD enzyme has significantly (P ≤ 0.05) increased as a result of Cu supplementation. The birds in G3 and G4 showed higher levels of SOD enzyme activity relative to birds in G2. These findings are consistent with previous studies

which have demonstrated an elevation in SOD's enzymatic activity of broilers supplemented with Cu-NPs compared to those provided with Cu_2SO_4 in their diets (Nassiri and Ahmadi, 2015; Ognik *et al*., 2017; El-Kassas *et al*., 2019; Ognik *et al*., 2019; Kazaz and Hafez, 2020). The unchanged TAC and MDA levels although the Cu- containing SOD enzyme has been significantly ($P \leq 0.05$) influenced could be a result of the fact that the antioxidant defense system includes numerous mechanisms, in which the role of the SOD enzyme comes within the third strategy after attempts to reduce the free radicles formation and to maintain the intactness of mitochondria, respectively (Surai *et al*., 2017; Surai *et al*., 2019). The stimulated activity of SOD as a result of Cu-NPs supplementation can undoubtedly be explained by the increased bioavailability of Cu-NPs over Cu_2SO_4 which could be confirmed by our results that indicated a higher Cu concentration in serum, liver, and breast muscle of birds supplemented with Cu-NPs in comparison to their counterparts fed on diets contain the same quantity of Cu_2SO_4. Many authors have noticed an increment in Cu deposition inside the tissues of animals supplemented with Cu-NPs (Scott *et al*., 2017a). The upregulation of MT in the liver of broiler chickens in G3 compared to others could be defined as a response to a higher load of Cu inside the enterocytes as a result of the dietary supplementation of 7.5 mg/kg of Cu-NPs relative to the same amount of Cu_2SO_4 or 3.75 mg/kg of Cu-NPs. Likewise, da Cruz Ferreira Júnior *et al*. (2022) have reported a linear relationship between Cu levels in the diets and the expression of MT in the liver of broiler chickens when they have compared the effects of the dietary supplementation of five levels of Cu (0, 4, 8, 12, and 16 mg/kg). This relationship could further confirm the higher bioavailability of Cu-NPs, where the modulation and binding to extra Cu are fundamental roles of MT (Thirumoorthy *et al*., 2011). The copper-binding transcription factor (Cup2p) specifically promotes the transcription of MT as a cellular response against Cu overloading (González *et al*., 2008).

CONCLUSION

The dietary supplementation of Cu-NPs as an alternative to common Cu_2SO_4 may safely enhance the physiological functions of Cu and the growth performance of broiler chickens. Cu-NPs could also allow a reduction of the used quantity and subsequently prevent the environmental burdens associated with Cu_2SO_4.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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