

ORIGINAL ARTICLE

Evaluation of copper nanoparticles and copper sulfate effect on immune status, behavior, and productive performance of broilers

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ABSTRACT

Objective: This study was conducted to compare between the nanoparticles of copper (Cu-NP) and copper sulfate (CuSO₄) effect on immunity, pro-inflammatory cytokine, oxidant/antioxidant balance, different behavioral patterns, growth rate, and weight gain by adding them in drinking water of broilers.

Materials and Methods: One hundred and fifty broiler chicks of 1-day-old were randomly divided into three groups: the first group was the control, the second group received CuSO₄ in drinking water (10 mg/l), and the third group received Cu-NP in drinking water (10 mg/l) with replicates (*n* = 25) for 5 weeks of age. Blood samples collected for estimating immunoglobulins (A, G, and M), superoxide dismutase, pro-inflammatory (IL-6), erythrocyte sedimentation rate, heterophil/lymphocyte (H/L) ratio, malondialdehyde (MDA), corticosterone hormone, and lymphoid organs index weight. Moreover, behavioral observations were undertaken from the 2nd week until the 5th week of age for measuring different behavioral patterns (feeding, drinking, crouching, body care, and comfort behaviors). Chicks were individually weighed weekly (gm); also, the weight gain, the intake of food, and the ratio of feed conversion were calculated.

Results: Cu-NP administration has improved the blood profile, lymphoid organs index weight in Cu-NP treated broilers in relation to control and CuSO₄ treated groups. Conversely, erythrocyte sedimentation rate, H/L ratio, MDA, and corticosterone hormone were reduced by Cu-NP treatment. Moreover, Cu-NP has a positive effect on all behavioral patterns more than other groups which significantly reflect on the weight of the body, gaining of weight, and the intake of food in the Cu-NP treated group of birds.

Conclusion: The findings in this study stated that when the drinking water of broilers was supplemented with Cu-NP, there might be an improvement in the immunity, behavior, and productive performance more efficiently than CuSO₄.

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Introduction

Copper (Cu) is an important mineral for chickens as it is essential for proper physiological functions; it is considered essential to hemoglobin synthesis, oxygen transport, and enzyme-coenzyme catalytic reactions [1]. Copper is involved in numerous physiochemical processes; in many enzymes, Cu is part of their active sites, including ascorbate oxidase, copper-zinc superoxide dismutase (CuZn-SOD), L-lysine oxidase, cytochrome c oxidase, dopamine beta-hydroxylase, and tyrosinase [2]. These enzymes are essential for antioxidant defense, melanin synthesis,

dopamine metabolism, mitochondrial respiration, and formation of connective tissue [3]. Cu plays an important role to inhibit inflammation by the synthesis of prostaglandins and converting arachidonic acid. Moreover, it affects the productive performance of animals as it acts as growth promoters [4]. However, excess amount of Cu in the animal diet has an adverse effect on growth and feed conversion as it result in low digestibility of Cu salt which does not exceed 20% and absorption in poultry which led to increased excretion of fecal matter and environment pollution [5]. Some researchers stated that

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Cu salts show less bioavailability than nano-sized Cu [6]. Also, copper effects on the function of living organisms as the mechanism of copper particles work are by affecting disease-causing microorganisms that have a direct effect on the animal's growth performance [7]. Also, it is used as an alternative for antibiotics that effects on chicken performance development by influencing the metabolism of nutrients as it affects the immune system which resulted in metabolic changes which known as immunological stress [8]. Animals are usually put in large groups for the production of food with high densities and are raised rapidly to slaughter.

Behavior is the way in which an animal interacts in response to a particular stimulus in its environment and its means of using available resources. In any given situation, both welfare and productivity are dependent upon behavior [9]. Methods of stress evaluation in poultry have been questioned by Hill [10] who stated that stress involves physical aspects and welfare deals with physiological aspects. For animals to adapt with changes in their social and physical environments, a buffering system is necessary, which is formed from complex behavioral, genetic and physiological responses.

The objective of the current study was to investigate the effects of the drinking water administration of Cu-NP or CuSO_4 on immunity status, hematological profile, pro-inflammatory cytokine, oxidant/antioxidant balance, behavioral patterns (ingestive, resting, movement activities, and comfort behavior), and productive performance (weight of the body, weight gain, intake of food, and ratio of feed conversion) of broilers.

Materials and Methods

Colloids

Colloidal Cu-NP solution (10 mg/l) of a particle size <100 nm, 99.8% trace metals basis, was purchased from Sigma-Aldrich Ltd., New York. The solution was manufactured from high purity metals (99.9999%) and demineralized water with high purity and mixed using a magnetic stirrer. The CuSO_4 (particle size: 106 μm was purchased from Sigma-Aldrich Ltd., New York, USA) solution was diluted in demineralized water before use.

Birds' management

One hundred and fifty chicks of 1-day-old collected from commercial broiler strain (Cobb 500) were allocated by weight (to reduce variation in average body weight) as birds weighed individually before dividing them in pens as the average weight of chicks were 42.7 ± 0.89 . Birds were randomly chosen from Animal Husbandry and Animal

Wealth Development Department; Faculty of Veterinary Medicine; Alexandria University. Flock was assigned into floor litter pens with a fully controlled environmentally housing system. Chicks were kept in pens with eight birds/m² stocking density. One-day-old chicks were subjected in the 1st week to brooding temperature (34°C) then gradually decline by 3°C/week until it reached 24°C by the end of the experiment. Artificial lighting was used to maintain lit conditions for 21 h per day during the experimental period. In accordance with feeding standards for poultry [11] (Table 1), they received ad libitum complete feed mixtures appropriate for the rearing period and had permanent access to drinking water.

The conditions in all pens were identical for all groups including floor space and light intensity. The protocol for this study was in compliance with the guidelines for the care of poultry and animals from the National Research Council and approved (2018/013/318) by the local commission of Ethics for the Care and Use of Laboratory Animals in Alexandria University, Egypt. Each step of this experimental design was conducted with the minimal pain or distress of the birds.

Experimental design

One hundred and fifty chicks of 1-day-old were allocated into three groups (25 birds/group) with replicate for each group with the same number of birds, group 1 control not receiving additives, group 2 received copper sulfate (CuSO_4) in drinking water (10 mg/l), and group 3 received copper nanoparticles (Cu-NP) (10 mg/l) in drinking water with other three groups' replicates. The doses were chosen because they were reported to be the best doses to enhance the blood profiles, biochemical parameters, metabolism, and performance in broiler chicken [12–14].

Blood and organ sampling

At day 35 (the end of the study), two blood samples were collected from 10 birds/group and the same number were taken from replicates that were selected randomly via puncture of the right wing vein. One blood sample was collected on heparin (anticoagulant) for hematological measurements; another blood sample was collected in a non-heparinized tube and left for 30 min till blood clotting then centrifuged for 10 min at 3,000 rpm with 4°C and the serum was separated for more analysis and kept at -70°C. The broilers were slaughtered and their total body weights were recorded. The lymphoid organs (spleen, thymus, and bursa of Fabricius) were removed carefully and weighed individually. The percentage of live body weight which was lastly recorded at the end of the experiment was used to calculate the relative weights of the lymphoid organs.

Table 1. Ingredients and calculated composition of basal non-supplemented diets for broilers.

Ingredients (%)	Starter	Grower	Finisher
Yellow corn	56.20	62.3	66.41
Corn gluten meal	6.00	3.70	2.00
Soybean oil meal (47%)	31.7	27.61	25.21
Soybean oil	2.30	2.50	2.50
DCP ^a	1.50	1.50	1.50
Limestone ^b	1.40	1.40	1.40
Mineral premix ^c	0.10	0.10	0.10
Vitamin premix ^d	0.10	0.10	0.10
Salt	0.30	0.30	0.30
DL-methionine ^e	0.20	0.22	0.22
Lysine HCl ^f	0.15	0.21	0.21
Choline chloride	0.05	0.05	0.05
Calculated composition			
Crude protein (%)	23.0	20.13	18.27
Calcium (%)	0.92	0.91	0.90
Available phosphorus (%)	0.47	0.46	0.46
Lysine (%)	1.21	1.15	1.08
Methionine (%)	0.59	0.56	0.52
ME (MJ/kg)	12.51	12.63	12.74
Energy/protein ratio ^h	0.543	0.627	0.697

^aDCP = Dicalcium phosphate (contain 18% phosphorus and 21% calcium).

^bLimestone (contain 35% calcium).

^cMineral premix: formulated and composed of (each 1 kg) 110,000 mg Mn (Mn oxide), 105,000 mg Zn (Zn oxide), 15,500 mg Cu copper oxide, 30,000 mg Fe (Fe sulfate), 1,125 mg I (sodium iodide), 325 mg Se (sodium selenite), 100 mg Co (cobalt sulfate), and calcium carbonate as a carrier.

^dVitamin premix composed (per 1 kg) of vitamin A 12,000,000 IU, vitamin D3 2,500,000 IU, vitamin E 10,000mg, vitamin K3 1000 mg, vitamin B1 1000 mg, vitamin B2 5000 mg, vitamin B6 1500 mg, vitamin B12 10 mg, niacin 30,000 mg, biotin 50 mg, folic acid 1000 mg, pantothenic acid 10 mg, and antioxidant ethoxyquin 250 mg with calcium carbonate as carrier.

^eDL-Methionine (Produced by Evonic Co. and contains 99% methionine).

^fLysine = lysine hydrochloride (contain 98% Lysine).

^gcalculated composition according to NRC (1994).

^hEnergy/protein ratio = ME KJ/CP%.

Hematological measurements

Within the first 1–2 h of collection, the blood samples were analyzed, for PCV (packed cell volume), Hb (hemoglobin) concentration, RBC (red blood cell), WBC (white blood cell) count, and a differential leukocytic count was performed [15]. The rate at which RBCs settle at unclotted blood in 1 h in the Wintrobe tube was used to determine ESR (erythrocyte sedimentation rate) in the blood [16]. The H/L (heterophil/lymphocyte) ratio was measured by dividing the number of heterophils by lymphocytes.

Immunological measurements

Serum immunoglobulins (IgA, IgG, and IgM), pro-inflammatory cytokine [interleukin (IL)-6], and lysozyme were estimated [17–19] using kits from Elabscience

Biotechnology Co., Ltd. (Houston, TX) in an enzyme-linked immunosorbent assay (ELISA) reader following the manufacturer instructions.

Copper, corticosterone and oxidant/antioxidant parameters

Using an atomic absorption spectrometer (Z-5300 Polarized Flame Atomic Absorption Spectrophotometer, Hitachi-Science & Technology, Tokyo, Japan), the Cu measurement in serum was performed. The method is based on the absorption of radiation by free metal atoms at a specific wavelength (324.8 nm for Cu). Analysis of corticosterone hormone based on the principle of photometric detection was performed using a commercial corticosterone indirect ELISA kit (via IBL international GmbH, Flughafenstrasse 52A, 22335 Hamburg, Germany). In the serum, MDA

(malondialdehyde) level and SOD activity were estimated using a Ransod diagnostics kit from Randox (London, UK) according to the manufacturer's instruction.

Behavioral observation

Behavioral observation was carried out according to the recommendations of Martin and Bateson [20] by scanning continuously with the naked eye. Observations were undertaken from the 2nd week until the 5th week of age. Behaviors of birds were recorded using observation with naked eyes and scan sampling. Behavioral observation was started at 6:00 a.m. till 6:00 p.m. during all day light 4 days per week, 3 h per day. Every hour of observation was divided into 5-min intervals scanning all birds and then starting with a new 5 min scan of all behaviours until the end of the observation time. The behavioral patterns observed were feeding, drinking, standing, crouching, walking, body care behavior (preening, ruffling, and shaking), and comfort behavior (wing stretch, leg stretch, and wing and leg stretch). All behaviors recorded are defined in Table 2. Results are expressed as the % of birds performing the categorized behavior/total number of birds observed [21].

Productive performance

During the course of the experiment, birds were weighed individually weekly (gm) for determination of weight gain of the body and feed conversion ratio (FCR), and also feed intake was recorded. FCR was calculated by dividing total feed intake (gm) by total weight gain (gm) of the broilers.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), means were compared by Duncan's test using the SAS statistical package [22]. For all variables immune

status, blood profile, lymphoid organ weight, behavioral patterns, and productive performance (weight of the body, weight gain, the intake of food, and conversion ratio of food), one-way ANOVA was used for the analysis. Duncan's test was used for post hoc analyses if treatment effects were significant. The overall significance level was set as $p < 0.05$. All values are expressed as the mean \pm standard error. Statistical model: $X_{ij} = \mu + T_i + e_{ij}$, where X_{ij} = Value of i th observation (the variable as body weight or behavior observation) of the i th treatment, μ = overall mean, T_i = effect of i th treatment, and e_{ij} = random error. Behavioral data were presented in the proportion of birds performing different behavioral activities.

Results

Lymphoid organs relative weight

As revealed in Table 3, the administration of Cu-NP (10 mg/l) to the drinking water of broilers led to a significant ($p < 0.05$) enhancement in the lymphoid organs relative weight compared to control and CuSO_4 (10 mg/l) treated group values. CuSO_4 also significantly ($p < 0.05$) increased the spleen and thymus relative weight, while the bursa of Fabricius relative weight had a non-significant increase relative to the control.

Hematological parameters of broilers

As defined in Table 4, PCV, Hb, and RBCs count showed a significant ($p < 0.05$) increase in Cu-NP treated broilers compared to their values in control and CuSO_4 treated group. On the contrary, WBCs count, ESR, and H/L ratio were reduced significantly in the Cu-NP group relative to the control and CuSO_4 groups. A significant ($p < 0.05$) enhancement was recorded in the CuSO_4 group PCV, Hb, and RBCs count, and a significant reduction in the WBCs count and ESR values compared to the control. While the H/L ratio revealed a non-statistical decrease in CuSO_4 group relative to the control.

Immune parameters in plasma of broilers

Treatment of broilers with Cu-NP led to a significant ($p < 0.05$) enhancement in IgA, IgG, and IgM values relative

Table 2. Ethogram of the recorded behaviors.

Behavior	Description
Feeding	Pecking at feed on feed troughs.
Drinking	Obtaining water from the cup.
Standing idle	The bird remains standing and does not perform any activity
Crouching	The bird remains sitting and lying on the litter looking about or with closed eyes, no other behavior.
Walking	Taking at least two successive steps.
Preening	The bird cleans and aligns its feathers using the beak.
Ruffling	Action of ruffle or shack all body feathers.
Shaking	Action of shaking body.
Wing stretch	The bird stretches one wing or both.
Leg stretch	The bird stretches one leg.
Wing and leg stretch	The bird stretches one leg and one wing of the same body hemisphere.

Table 3. Effect of copper sulfate (CuSO_4) and copper nanoparticles (Cu-NP) on the lymphoid organs relative weight (g/kg) of broilers.

	Spleen	Thymus	Bursa of Fabricius
Control	0.105 \pm 0.003 ^c	0.324 \pm 0.007 ^c	0.051 \pm 0.001 ^b
CuSO_4	0.121 \pm 0.002 ^b	0.361 \pm 0.004 ^b	0.056 \pm 0.001 ^{ab}
Cu-NP	0.145 \pm 0.005 ^a	0.424 \pm 0.011 ^a	0.067 \pm 0.002 ^a
<i>p</i> -value	0.0014	0.0012	0.013

Means bearing different letters within the same column are significantly different ($p < 0.05$). Data were shown as mean \pm SE.

to the control. Also, the CuSO₄ treated birds revealed a significant ($p < 0.05$) enhancement in IgA and IgM, and a non-significant increase in IgG compared to the control. IL-6 and lysozyme revealed a non-significant but numerical change in their values between the three groups of broiler chickens (Table 5).

Copper, corticosterone, and oxidant/antioxidant parameters in plasma

The findings of the present study (Table 6) revealed that Cu-NP treatment significantly ($p < 0.05$) enhanced copper and SOD levels in broilers when compared to the control and CuSO₄ treated birds. On the contrary, corticosterone and MDA were reduced significantly ($p < 0.05$) in the Cu-NP group relative to the control and CuSO₄ treated group. Also, the CuSO₄ treated group had a significant ($p < 0.05$) enhancement in copper and SOD levels, and a significant decrease in corticosterone and MDA compared to the control.

Behavioral observation

The effects of CuSO₄ and Cu-NP on behavioral patterns of broilers are summarized in Table 7. Results showed that

birds having Cu-NP significantly exhibit more ingestive behavior (feeding and drinking) than those having CuSO₄ ($p < 0.01$). Moreover, crouching, comfort behavior (wing stretch, leg stretch, and wing and leg stretch), and body care behavior (preening and ruffling) were significantly higher in the nano copper group than CuSO₄ and control groups ($p < 0.01$). Contrarily, standing idle and walking behaviors were significantly higher in the control group than the other two groups. While no significant variation in shaking behavior between CuSO₄ and Cu-NP groups ($p < 0.023$) but it was significantly higher than the control group.

Productive performance

Copper supplementation affected final body weight relative to the control (Table 8). The initial BW at 14 days was adjusted to avoid differences between the groups and final body weight was at 35 days. The highest body weight was observed in group 3 administered Cu-NP in drinking water than birds received CuSO₄ and the lowest body weight was significantly found in the control group ($p < 0.001$).

Weekly weight gain and total weight gain were calculated from the recorded data shown in Table 9. The highest

Table 4. Effect of copper sulfate (CuSO₄) and copper nanoparticles (Cu-NP) on hematological parameters of broilers.

	PCV (%)	Hb (g/dl)	RBCs (10 ¹² /l)	WBCs (10 ⁹ /l)	ESR (mm/h)	H/L ratio (%)
Control	25.42 ± 0.27 ^c	13.23 ± 0.04 ^c	2.21 ± 0.01 ^c	25.4 ± 0.26 ^a	3.85 ± 0.02 ^a	0.523 ± 0.01 ^a
CuSO ₄	26.61 ± 0.24 ^b	14.87 ± 0.06 ^b	2.41 ± 0.02 ^b	24.6 ± 0.32 ^b	2.97 ± 0.01 ^b	0.479 ± 0.02 ^{ab}
Cu-NP	27.35 ± 0.31 ^a	15.35 ± 0.08 ^a	2.92 ± 0.01 ^a	23.1 ± 0.38 ^c	2.52 ± 0.01 ^c	0.452 ± 0.07 ^b
<i>p</i> -value	0.0001	0.0001	0.0018	0.0004	0.0001	0.041

Means bearing different letters within the same column are significantly different ($p < 0.05$). Data were shown as mean ± SE. PCV: Packed Cell Volume; Hb: hemoglobin; RBCs: Red Blood Cells; WBCs: White Blood Cells; ESR: Erythrocyte Sedimentation Rate; H/L ratio: Heterophil/Lymphocyte ratio.

Table 5. Effect of copper sulfate (CuSO₄) and copper nanoparticles (Cu-NP) on the immune and pro-inflammatory cytokine parameters in the serum of broilers.

	IgA (g/L)	IgG (g/L)	IgM (g/L)	IL-6 (pg/ml)	Lysozyme (μg/l)
Control	0.062 ± 0.002 ^c	0.089 ± 0.003 ^b	0.092 ± 0.005 ^b	5.62 ± 0.07	3.224 ± 0.04
CuSO ₄	0.088 ± 0.002 ^b	0.115 ± 0.006 ^{ab}	0.128 ± 0.007 ^a	5.54 ± 0.06	3.116 ± 0.02
Cu-NP	0.103 ± 0.003 ^a	0.134 ± 0.008 ^a	0.148 ± 0.009 ^a	5.58 ± 0.04	3.105 ± 0.02
<i>p</i> -value	0.001	0.001	0.001	0.2539	0.5241

Means bearing different letters within the same column are significantly different ($p < 0.05$). Data were shown as mean ± SE. IgA, G, and M: immunoglobulins A, G, and M; IL-6: Interleukin-6.

Table 6. Effect of copper sulfate (CuSO₄) and copper nanoparticles (Cu-NP) on copper, corticosterone, and oxidant/antioxidant parameters in the serum of broilers.

	Cu (μg/l)	Corticosterone (ng/ml)	MDA (μmol/l)	SOD (U/ml)
Control	114.8 ± 6.3 ^c	4.95 ± 0.081 ^a	1.748 ± 0.027 ^a	1.532 ± 0.031 ^c
CuSO ₄	159.2 ± 5.9 ^b	3.26 ± 0.065 ^b	1.137 ± 0.021 ^b	1.724 ± 0.029 ^b
Cu-NP	168.4 ± 8.7 ^a	2.74 ± 0.027 ^c	1.016 ± 0.011 ^c	1.965 ± 0.047 ^a
<i>p</i> -value	0.001	0.0001	0.0042	0.0015

Means bearing different letters within the same column are significantly different ($p < 0.05$). Data were shown as mean ± SE. Cu: Copper; MDA: Malondialdehyde; SOD: Superoxide Dismutase.

Table 7. Effect of copper sulfate (CuSO₄) and copper nanoparticles (Cu-NP) on behavioral patterns of broilers.

	Control	CuSO ₄	Cu-NP	p-value
Feeding	9.74 ± 0.58 ^c	11.89 ± 0.94 ^b	13.71 ± 1.01 ^a	0.0001
Drinking	7.31 ± 0.65 ^c	9.22 ± 0.82 ^b	12.98 ± 0.89 ^a	0.0001
Standing	8.87 ± 0.78 ^a	5.11 ± 0.23 ^b	3.78 ± 0.89 ^c	0.0001
Crouching	68.43 ± 5.12 ^b	82.62 ± 4.05 ^{ab}	85.52 ± 6.57 ^a	0.0245
Walking	14.48 ± 0.72 ^a	9.23 ± 0.83 ^c	7.89 ± 0.99 ^b	0.001
Preening	3.64 ± 0.42 ^c	6.79 ± 0.59 ^b	8.23 ± 0.25 ^c	0.0001
Ruffling	4.70 ± 0.03 ^b	5.28 ± 0.21 ^{ab}	5.78 ± 0.34 ^a	0.026
Shaking	4.23 ± 0.15 ^b	5.06 ± 0.27 ^a	5.59 ± 0.43 ^a	0.023
Wing stretch	3.19 ± 0.36 ^c	7.62 ± 0.63 ^b	8.53 ± 0.74 ^a	0.0001
Leg stretch	3.50 ± 0.03 ^c	5.34 ± 0.18 ^b	9.16 ± 0.48 ^a	0.0001
Wing and leg stretch	5.76 ± 0.52 ^c	7.23 ± 0.23 ^b	9.44 ± 0.49 ^a	0.0001

Means bearing different letters within the same column are significantly different ($p < 0.05$). Data were shown as mean ± SE.

Table 8. Effect of copper sulfate (CuSO₄) and copper nanoparticles (Cu-NP) on body weight (gm) in broilers aged 14, 21, 28, and 35 days.

	14 days	21 days	28 days	35 days
Control	415.23 ± 4.32	855.78 ± 15.53 ^{ab}	1263.17 ± 31.49 ^c	1584.50 ± 25.94 ^c
CuSO ₄	420.77 ± 4.94	876.93 ± 13.26 ^b	1379.39 ± 21.11 ^b	1798.23 ± 48.72 ^b
Cu-NP	425.13 ± 5.57	897.74 ± 29.61 ^a	1490.25 ± 35.81 ^a	1983.65 ± 67.44 ^a
p-value	0.28	0.02	0.0001	0.0001

Means bearing different letters within the same column are significantly different ($p < 0.05$). Data were shown as mean ± SE.

Table 9. Effect of copper sulfate (CuSO₄) and copper nanoparticles (Cu-NP) on the productive performance of broilers.

	WG1	WG2	WG3	TWG	FI	CFCR
Control	440.55 ± 13.86 ^b	407.39 ± 21.56 ^c	321.33 ± 17.06 ^c	1169.27 ± 31.56 ^c	601.35 ± 14.78 ^c	2.57 ± 0.05 ^a
CuSO ₄	456.16 ± 15.76 ^{ab}	502.46 ± 24.74 ^b	418.84 ± 37.32 ^b	1377.46 ± 43.83 ^b	667.82 ± 12.42 ^b	2.42 ± 0.04 ^b
Cu-NP	472.61 ± 19.68 ^a	592.51 ± 25.49 ^a	493.41 ± 47.62 ^a	1558.52 ± 67.62 ^a	750.54 ± 16.32 ^a	2.41 ± 0.06 ^b
p-value	0.0253	0.001	0.001	0.0001	0.001	0.007

Means bearing different letters within the same column are significantly different ($p < 0.05$). Data were shown as mean ± SE. WG1, weight gain from 14 to 21 days (gm); WG2, weight gain from 21 to 28 days (gm); WG3, weight gain from 28 to 35 days (gm); TWG, Total weight gain from 14 to 35 days (gm). FI, feed intake (gm/week). CFCR, cumulative feed conversion ratio.

weight gain was observed in group 3 of Cu-NP in the first, second, and third weeks of the study. The total body weight gain values were also significantly different between the three groups, with the highest value recorded in group 3. Moreover, birds in group 3 had a higher intake of food relative to the other groups. While there were no significant variations in the conversion ratio of food between the Cu-NP group and CuSO₄ group, as the highest conversion of food was observed in the control one.

Discussion

Improvement in poultry physiological condition and performance after treatment with copper nanoparticles

mechanisms are still not fully clear. So, the goal of this work was to study the effects of administration of Cu-NP or CuSO₄ (10 mg/l) in the drinking water on the immune status, blood profile, pro-inflammatory cytokine, behavioral patterns (ingestive, resting, movement activities, comfort, and body care behavior), and productive performance (body weight and weight gain) of broiler chicken.

The spleen, thymus, and bursa of Fabricius (immune organs) have a very important role in the immune response against harmful substances of chickens, particularly the spleen [23].

One of the very important parameters for the immune status of the birds is the immune organ indexes which are the ratios of immune organ weights to body weight. In the

current experiment, all the lymphoid organ indexes were enhanced by adding Cu-NP; which indicate an improved immune status of the broilers after the addition of Cu-NP in the drinking water. Our findings were consistent with the previous results reported by Wang et al. [17] who also recorded an increase in the lymphoid organs (bursa of Fabricius, spleen, and thymus) index weight after the supplementation of copper-loaded chitosan nanoparticle (CNP-Cu) in the diet of broilers.

Many literatures revealed that copper has a significant role in RBC production, synthesis of Hb, and also iron metabolism [1,2] as ceruloplasmin which also takes part in iron metabolism, by transporting about 95% of Cu contained in blood [24]. Mediated by the transferrin receptor, Fe^{2+} will be oxidized to Fe^{3+} , which enables the binding of iron to transferrin and it is an important mechanism for the transportation of iron from the blood into the cells; also, iron helps in the transportation of oxygen by hemoglobin [25]. That is why the hematological parameters changes were recorded in Cu-NP research studies in poultry [13,26]. Our findings agree with the previous studies of Ognik et al. [2] that reported an increase in PCV, Hb, and RBCs count and reduced WBC count after drinking water administration of Cu-NP of broiler chickens. In another experiment [13], after intramuscular injection of Cu-NP or Cu microparticles; enhanced RBCs count and Hb were recorded in chickens; the authors also revealed that according to the size of the Cu particle, the rate of erythropoiesis was dependent. On the contrary, chickens receiving Cu-NP had a decrease in hemoglobin content in the blood [26]. However, the obtained blood parameter values in our study were within the normal (physiological) range for chickens [27].

In the current study, the used dose of Cu-NP did not alter the value of the pro-inflammatory cytokine (IL-6) and thus was well tolerated by the birds; which means that it did not cause any inflammatory reaction. In a previous study [2], which increased the administration of copper in the diet to the way much above the NRC [11] recommendations, blood IL-6 increased in the chickens; that was accompanied by an increase in ceruloplasmin activity following the treatments.

In our experiment, the administration of Cu-NP led to inhibition of other inflammatory markers which is ESR and H/L ratio. An enhancement in the level of pro-inflammatory cytokines (including IL-6, C-reactive protein, and ceruloplasmin) will be noted with any inflammatory reaction, which is important for restoring homeostasis to normal physiological ranges [28,29]. The faster ESR and enhanced H/L ratio will take place due to the changes during inflammation in the proportions of serum proteins (an increase in globulins and fibrinogen and a decrease in albumins). Since the addition of Cu-NP to the drinking

water of broilers, in our study, did not increase IL-6, so the ESR value and H/L ratio did not increase as well but they were even reduced indicating possible anti-inflammatory properties of Cu-NP [17].

Our results showed that with Cu-NP administration, immunoglobulins (IgA, IgG, and IgM) enhanced while lysozyme activity did not alter in the bird's blood. The improvement in immunoglobulins concentration can furthermore activate many complements and so it could stimulate the bird's immunity and so help the birds to fight against many morbid diseases [23]. These results are in consistent with the findings reported by Wang et al. [17] who reported that the addition of Cu-NP to chickens in the feed caused enhancement in the level of IgA, IgG, and IgM. Also, it was reported that an increase in the values of immunoglobulin's (IgA, IgM, and IgY) with increasing the dosage of Cu-NP [2]. On the contrary, there were no changes in the levels of IgG or IgM in the broilers blood after injecting a hydrocolloid of Cu-NP into the air chamber of the fertilized eggs of chickens [12]. In our experiment, increased immunoglobulin's values may be due to activation of B lymphocytes as a result of direct reaction with the Cu-NP or indirectly induced by cytokines released from macrophages or other phagocytes. It could also be explained that the immunoglobulin's enhancement levels may have been the result of the activation of phagocytes which indicate an improved immune status of broilers after Cu-NP treatment [2].

An essential step in the studies related to poultry production is the detection of oxidative stress damage and the protection against it by anti-oxidants; as the values of lipid peroxidation and antioxidant parameters act as an indicator for the metabolic status of the organism rather than metabolic parameters alone [5,19]. Summarized studies in a review indicate that Cu in the diet with moderately high levels seem to affect lipid metabolism, by decreasing cholesterol values in the poultry products [8]. Another effect of Cu in the diet of animals is that it has an antioxidant protection [2,12]. By injecting hydrocolloid of Cu-NP in the fertilized egg air chamber, it may decrease the intensity of oxidative processes in growing chickens [12]. The results of our experiment revealed that the treatment with nanoparticles of Cu caused an enhancement in the serum SOD activity and reduced content of MDA indicating that the administration of Cu-NP suppressed the oxidative stress in the broilers. Generation of harmful free radicals occurs due to severe lipid and protein oxidation process, which may lead to a reduction of endogenous antioxidants and an increase at first, followed by a reduction, in the activity of antioxidant enzymes [30]. This explanation is reflected in our work with the increase recorded in SOD activity and the low level of MDA in the Cu nanoparticles administered group. Copper in the blood or tissues may

exhibit protective properties against oxidative stress when the body's redox balance is disturbed [31].

Serum corticosterone level has been mostly used as the most reliable marker for acute stress in chickens [32]. In the current work, the addition of Cu-NP in broilers drinking water led to the reduction of serum corticosterone value; which might indicate an inhibition of the stress condition in the treated chickens. Exogenous corticosterone supplementation which acts as a stress condition in poultry has enhanced the lipid peroxidation levels [33]. Since the oxidant/antioxidant status in the Cu-NP treated broilers was improved and the immunological stress condition was minimized, so the serum corticosterone level was reduced. In the current study, all the values of hematological, immunological, and oxidant/antioxidant parameters improvement, after Cu-NP administration, were more beneficial than the improvements induced by copper sulfate treatment, which might be due to the enhanced uptake of the nanoparticles by the organism more than the regular micro-particles [34]. These findings are also in correlation with the increased Cu level in the serum of Cu-NP treated group more than the CuSO₄ treated group, in the current work.

There is little quantitative data available for comparing the effects of CuSO₄ and Cu-NP treated groups of broilers on the subsequent behaviors. Results showed that there was a highly significant effect of adding nanoparticles on the ingestive behavior of birds (feeding and drinking) in group3 than other groups as it was the highest in the Cu-NP group and the lowest in the control group. In general, birds that receive Cu in nano form or in inorganic form show an increase in ingestive behavior. These finding may be attributed to Cu is a growth promoter that enhances feed intake and so it affects directly on ingestive behavior by increasing it. Also, the cause that ingestive behavior is higher in Cu-NP than CuSO₄ is copper nanoparticle have higher uptake in the gastrointestinal tract (GIT) [35,36], so it doubles its effect as growth promoter than CuSO₄. Moreover, crouching behavior percent was significantly higher in the Cu-NP group than in other groups. Also, comfort behavior (wing stretch, leg stretch, and wing and leg stretch) and body care behavior (preening and ruffling of feather) were significantly higher in the nano copper group than CuSO₄ and control groups. Percent of birds exhibit those behaviors were higher in Cu-NP than CuSO₄ group or control group which indicate that birds are under the condition of welfare and they are not under stress condition, this explanation is obvious in the measurement of H:L ratio and corticosterone level as they are a more reliable indicator of stress [37]. Also, it was reported that H:L ratio decreased with decreasing stress levels on the birds [38,39]. Moreover, Al-Murani et al. [40] stated that stress leads to enhance

the number of heterophil and causes reduction in the number of lymphocytes and increases the H: L ratio. The physiological manifestations of stress in poultry include changes in the number of circulating leukocytes which led to heterophilia and lymphocytopenia which is one of the reliable indicators of stress. Similarly, Hester and Sheamoore [41] stated that heterophils and lymphocytes are more responsive to stressors than other leukocytes by making their differential changes less difficult to detect than others as they were less variable and more enduring than corticosterone response. The lowest level of H:L ratio and corticosterone hormone level was observed in the Cu-NP treated group. On the other hand, walking and standing idle were significantly higher in the control group than the other two groups. While there were no significant variations in shaking behavior between CuSO₄ and Cu-NP groups but it was significantly higher than the control group. A lot of information about the effect of Cu nanoparticles are not yet known, and, in our study, we monitor most of the behavioral patterns in broilers but it still needs further studies.

In our study, we found that Cu-NP had the highest live body weight, weight gain, and feed intake than birds received CuSO₄ and more than the control group. In the previous investigation, Cu-NP has an effect on the body weight of broilers than adding CuSO₄ [4]. Moreover, Cu administration *in ovo* could have prolonged effects on the productive performance of broiler as reported by Mroczek-Sosnowska et al [42]. Moreover, adding 50 and 100 mg/kg of Cu-loaded chitosan nanoparticles enhances the growth performance of broiler chickens [17]. Furthermore, intramuscular injection of Cu-NP improved broiler chickens growth [13]. In our results, final live body weight was improved in all treated groups compared with the control group. Similar to our results, it was reported Cu-NP supplementation significantly enhanced growth performance in piglets [6]. Furthermore, Cu-NP has similar effects on the final BW in fish [18].

These findings may be attributed to that Cu-NP had a more beneficial effect than CuSO₄, which might be due to the better uptake of Cu-NP in the GIT and the higher bioavailability of the nanoparticles. The small size of nanoparticles might result in faster diffusion through the GIT mucus to reach the cells of the intestinal lining, followed by uptake through the GIT barrier to reach the blood [36]. Also, Cu could enhance the enzymes activities involved in nutrient utilization making metabolic pathways more efficient [43]. Improvement in performance and growth might be due to increasing energy digestibility in animals treated with Cu-NP than with CuSO₄; also, the bioavailability of Cu-NP was higher than CuSO₄ when compared with each other [6]. Additionally, Cu could enhance the absorption of fat-soluble vitamins and fatty acids, thereby increasing the

metabolism of different nutrients and influencing broilers growth performance by an increase in lipase and phospholipase activity in the small intestine [43]. Moreover, the mechanism behind improving animal performance attributed to the improvement of antimicrobial and antibacterial properties of Cu-NP [44]. That is why Cu is always used as a growth promoter but in our results, we found that using Cu-NP is more beneficial than CuSO_4 as it enhances the growth-promoting effect of Cu.

Conclusion

It can be concluded that, from the above discussion, the results of the experiment reveal many-promising effects of Cu-NP. Administration of Cu-NP to the drinking water of broilers enhanced the physiological condition of the birds by improving the immune status, hematological parameters and stress-related hormone, enhanced the organism antioxidant potential, and reduced oxidative stress. Moreover, Cu-NP also increases bird's welfare by decreasing stress as well as all stress indicators, and birds are more free to exhibit their behavioral patterns which reflect on their productive performance and weight gain. All these impacts of Cu-NP were more beneficial than the addition of CuSO_4 in the drinking water of broilers; and so this study recommends the usage of Cu-NP for improving poultry performance and production; but also more studies are required to understand the full potential of mineral nanoparticles.

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Conflict of interest

The authors declare that they have no conflict of interest related to the publication of this paper.

Authors' contributions

This work was carried out in collaboration between all authors. SEK and MHH conceived the study and the design of the experiment and both carried out the fieldwork. MHH carried out all physiological, immunological, and hematological measurements and SEK carried out all behavioral patterns measurements and productive performance. All authors were involved in revising the manuscript and approved the final revision.

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