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The Impact of Alternative Dietary Replacement of Inorganic Copper Salt with Organic and Nano Form on Productive Performance and Egg Quality Characteristics of Laying Hens

Mohamed I. El-Katcha[1], Mosaad A. Soltan[1], Seham M. El-Kassas[2]*, Mahmoud M. Arafa[3], El-Sayed R. Kawarei [3], Karima M. El-Naggar[1]*

[1]Department of Nutrition and Veterinary Clinical Nutrition, Faculty of Veterinary Medicine, Alexandria University, 22758, Edfina, Egypt.
[2]Animal, Poultry and Fish Breeding and Production, Department of Animal Wealth Development, Faculty of Veterinary Medicine, Kafrelsheikh University, 33516, Kafrelsheikh, Egypt.
[3]Biochemistry, Toxicology and Deficiency Diseases, Animal Health Research Institute, El-Dokii, Giza, 12618, Egypt.

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MIE-K, MAS and MMA designed the work. MAS, MMA and ERK performed the experimental trial. ERK and KME-N collected the samples, ran the analysis and collected the data. SME-K analyzed the raw data and wrote the manuscript.

Key words
Laying performance, Egg production, Egg shell, Nano copper, Organic copper

ABSTRACT

This study investigated the effect of dietary replacement of inorganic copper (CuO) with its organic or nano source on productive performance, egg quality and blood biochemical constituents of layers. Three hundred, 55 weeks old of Isa Brown hens were randomly allotted into 5 groups. First group received CuO at 8 mg/kg diet, while the second and third group supplemented with the organic form of Cu (copper polysaccharide complex) at 8 and 4 mg/kg diet, respectively. Fourth and fifth group received the Cu as nanoparticles (CuO-NPs) at the same levels of the organic form, respectively. Birds fed the experimental diets for 10 weeks at 110 g/hen/day. Organic Cu failed to compensate body weight losses ($P > 0.05$), and the CuO-NPs significantly increased it ($P < 0.05$). Both organic and CuO-NPs non-significantly increased average egg production, shell composition of calcium and phosphorus % and yolk Cu contents ($P > 0.05$). In contrast, the organic and Nano-CuO salts reduced egg yolk cholesterol contents ($P < 0.05$). Besides, they caused an interesting reduction of cracked egg percentage ($P < 0.05$). Additionally, both organic and CuO-NPs increased serum Cu levels, MDA and SOD activities while reduced serum glutathione peroxidase activity compared with CuO ($P < 0.05$). Moreover, serum lipid profile was significantly altered as triglycerides concentration was increased while total cholesterol was reduced in birds received organic and CuO-NPs. So, the organic or Nano forms of Cu could be used as safe alternative sources in layer diets replacing the inorganic Cu without compromising their productive performance.

INTRODUCTION

Improving egg production and reducing its associated problems such as the low egg quality are the main objectives of the layer producers. Out of these problems, eggshell cracking is a serious problem directly related to a lower shell quality, which is influenced by many factors such as mal-nutrition, defective managemental procedures, bad environmental conditions, bird's age and genetics (Mazzuco and Bertechini, 2014). Besides that, the egg shell quality is commonly decreased during the declining phase of egg production when hens become older with a reduced ability to utilize calcium (Arifin, 2016). Therefore, improving these factors through the application of different approaches such as micronutrients supplementation have been taken into consideration to enhance eggshell quality (Nys, 2001; Roberts, 2004).

Copper (Cu) is a crucial micro-element and maintains growth and production of different animal species. It acts as a co-factor in numerous enzymatic systems such as lysyl oxidase, superoxide dismutase, and cytochrome oxidase, or ceruloplasmin which are required for eggshell formation (Abo-Al-Ela *et al*., 2021). Earlier studies reported that Cu is

* Corresponding author: seham.elkassas@vet.kfs.edu.eg, karima.muhammad@alexu.edu.eg
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important to increase shell strength of table eggs (Pekel and Alp, 2011) and reduce cholesterol content in the yolk (Lim and Paik, 2006). Therefore, its deficiency alters lysine-derived-crosslinks leading to malformation of eggshell because of the defective distribution of shell membrane fibers resulting in egg shape deformation and changing in its mechanical properties (Mazzuco and Bertechini, 2014). Additionally, its deficiency lowers egg production and increases the number of low quality eggs produced (Saleh *et al*., 2020). Commercially, inorganic forms of Cu such as Cu sulfate or Cu oxide are the common sources included in the diet formulation due to its cost and commercial availability (El-Kassas *et al*., 2020). But, unfortunately, these sources have low bioavailability, suffer from high rates of loss due to dietary antagonists which form insoluble compounds affecting Cu absorption and utilization inside the body (Aksu *et al*., 2012) and consequently increasing environmental concerns by higher heavy mineral excretion.

Therefore, there is an extensive interest in substituting inorganic trace minerals by more bioavailable sources that can meet bird requirements, improve its production as well as reducing mineral losses to the environment (Nollet *et al*., 2007). Organic or chelated source considered one of these alternative sources which showed several advantages over the inorganic ones such as protection from undesired chemical reactions in gastrointestinal tract, higher bioavailability and reduced mineral excretion (Li *et al*., 2005; Skřivan *et al*., 2010; Stefanello *et al*., 2014). Another important source newly emerged with the development of nanotechnology is the nanoparticle form of these minerals. These nanoparticles showed a high degree of transport and uptake inside the body and exhibit high absorption efficiency (Davda and Labhasetwar, 2002). Recently, Cu nanoparticles (Cu-NPs) attracted the attention as a promising alternative source included in animal diet (Gonzales-Eguia *et al*., 2009; Mroczek-Sosnowska *et al*., 2013; Muralisankar *et al*., 2016). However, there is a little information on its inclusion in layer diets and their effects on the productive performance and the eggshell characteristics. Therefore, the present study examined the effect of dietary replacement of inorganic Cu (CuO) with organic Cu (copper polysaccharide complex) or Cu oxide nanoparticles (CuO-NPs), at the same recommended levels of inorganic form or at lower levels, on egg production performance, egg quality, immune response, and some blood biochemical constituents of laying hens.

MATERIALS AND METHODS

Management, experimental design and feeding program of hens

Bird management procedures were approved and followed the requirements of local ethical committee of animal use, Faculty of Veterinary Medicine, Alexandria University, Egypt.

A total of 300, 55 weeks old Isa Brown laying hens were purchased from a commercial company (Hegazy Group Co, Cairo, Egypt) and used in this experiment. Birds were individually weighed and those with non-significant statistical differences in body weight were randomly divided into 5 separate groups with 4 replicate/ group (15 hens/ replicate). Each bird was individually caged in a cage of 45 × 40 × 65 cm^3 in size. Cages were placed in an open-sided poultry house. Temperature was maintained at 21 to 24°C with light cycle was adjusted at 16 h light/ 8 h darkness depending on natural and artificial light. Each cage was equipped with a nipple drinker and metal trough to ensure that each bird fed separately from the others. Birds received feed at a level of 110 g/hen/day and a free access to water throughout 10 weeks of experimental period.

The basal diet (BD) (Table I) was formulated according to the nutrient requirements of poultry (NRC, 1994). The inorganic Cu (CuO) was added at a concentration of 8 mg/kg diet according to the nutrition management guide of the Isa Brown breed. Organic Cu (copper polysaccharide complex), and CuO-NPs were added to the BD at 100% and 50% of the inorganic Cu. Therefore, the current experiment included five groups; group 1(G1) which received the inorganic Cu (Cu oxide, 8 mg/kg diet), group 2 and 3 (G2 and G3) received organic Cu (copper polysaccharide complex, Quali Tech, Chaska, MN with guaranteed minimum of 30% Cu, added at levels of 8 and 4 mg/kg diet, respectively) while group 4 and 5 (G4 and G5) received CuO-NPs (copper oxide nanoparticles produced by Mknano Co. M K Impex Corp, Canada with 50 nm particle size, 90% purity and added at the levels of 8 and 4 mg/kg diet, respectively (Fig. 1). Proximate chemical analysis of feed samples from different experimental groups was done according AOAC (1990).

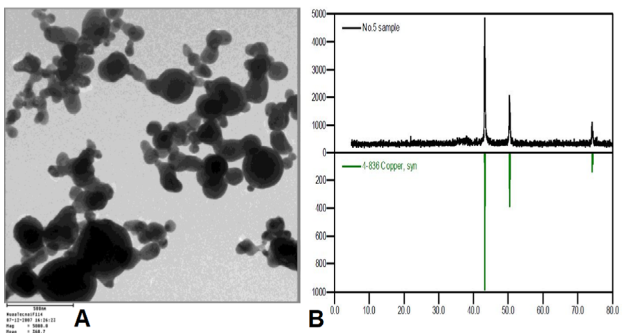

Fig. 1. Characteristics of CuO-NPs. A, represents the TEM images of the synthesized CuO particles with average size of the observed particles is ~ 50 nm. B, shows CuO-NPs powder XRD pattern.

Table I. Ingredients and chemical composition of the used basal diet.

Ingredients	g/kg
Yellow corn	578.8
Soybean meal (44% CP)	220
Corn gluten meal (60% CP)	40
Wheat bran	30
Sunflower oil	12.5
Ground lime stone[1]	98
MCP [2]	13
Common salt	2.5
Vitamin premix [3]	1.5
Mineral premix [4]	1
Lysine[5]	0.2
Methionine[6]	1
Choline chloride [7]	1
Mycotoxin adsorbent	0.5
Chemical composition (%)	
Moisture	11.65
Crude protein	17.07
Ether extract	4.06
Ash	12.76
Crude fiber	4.43
NFE[8]	50.03
Calcium	3.72
Phosphorus	0.63
Copper [9]	8 ppm
ME Kcal/kg[10]	2746.88

[1]Lime stone contains 37% calcium and locally produced. [2]Mono calcium phosphate: contain 21% phosphorus and 17% calcium. [3]Vitamin premix: Each 1.5 kg contains: Retinyl acetate (12000000 IU), Cholecalciferol (2000000 IU), Tocopherol acetate (10 g), Menadione (2 g), Thiamine (1g), Riboflavin (5g), Pyridoxine HCL (1.5g), Cyanocobalamin (10 g), Niacin (30 g), Pantothenic acid (10 g), folic acid (1g), biotin (50 mg), produced by Archar Daniels Midland company, IL, USA. [4]Mineral premix was formulated and composed of (1 kg): 70000 mg Manganese, 60000mg Zinc, 8000mg Copper, 50000 mg Iron, 1000 mg Iodine, 250 mg Selenium and 150 mg Cobalt and calcium carbonate up to 1 kg. [5]Lysine: 98% lysine hydrochloride, produced by Shandyoung Longue Co., China. [6]DL-methionine produced by Evonik Co. (99.5% DL-methionine). [7]Choline: choline chloride 60% with vegetable carrier (corn powder) produced by Shandyuong pharmaceutical Co., China. [8]Nitrogen free extract calculated by difference. [9]calculated without premix addition, [10]ME was calculated according to (NRC, 1994) .

Measurements of productive performance

Birds were weighed individually at the beginning and end of the 10 weeks feeding trial and the changes in live body weight (BW) were calculated. Eggs produced from various groups (on an individual basis) were collected daily to calculate % of hen day egg production (HDEP).

HDEP= Total number of produced eggs per period/ Total number of layers in the same period×100.

About 30 eggs per replicate from each group were randomly collected (every 14 days/ period for five successive periods) and weighed to calculate the average egg weight (EW).

EM= % HDEP × Average egg weight in gram.

Also, the amount of feed (FI) consumed to produce a unit of EM was recorded and feed conversion ratio (FCR) was calculated.

FCR= Feed consumed (Kg)/ egg produced (Kg)

The percent of cracked shell was recorded daily. A sample of 30 eggs from each replicate (n = 120 /group) was randomly collected at the middle and the end of experimental period (59th and 65th weeks of age) to estimate yolk and albumin weight and their relative weight to total egg weight, yolk and albumen index, shell thickness and weight as well as its relative weight according to Card and Nesheim (1972).

Random ten eggshell samples from each replicate (n = 40/ group) were wet-ashed with nitric acid (HNO_3) and perchloric acid for preparation of samples for calcium determination using flame photometer according to Slavin (1968) and phosphorus according to Gericke and Kurmies (1952). Egg yolk was dried, and then one gram of dried tissue was digested with 10 ml of HNO3 and used for determining Cu content in yolk by atomic absorption spectrophotometer. Egg yolk cholesterol was analyzed based on method described by (Rotenberg and Christensen, 1976).

Evaluation of immune response

Ten blood samples/ replicate (n= 40/ group) were randomly collected at the end of the experiment (65th weeks of hen's age) in clean dry vials containing anticoagulant (0.1ml sodium citrate 3.8%) for determination of phagocytic activity (PA) and phagocytic index (PI) according to Kawahara *et al*. (1991). Hemagglutination inhibition test (HI) to detect Newcastle and avian influenza antibodies was done according to Takatsy (1955). Briefly, blood samples (ten samples/ replicate) were randomly collected at the 59th and 65th week of laying period in clean dry vials without anticoagulant. Blood samples were kept coagulating and serum was separated by centrifugation at 3000 rpm for 10 min and kept at -20 °C for further analysis. Geometric mean titer (GMT) was calculated according to Brugh (1978).

Blood biochemical parameters

Biochemical constituents including triglycerides, total cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL), glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), uric acid, creatinine, superoxide dismutase (SOD), glutathione peroxide (GPx) and lipid peroxidase (Malondialdehyde “MDA”) and some minerals as calcium, phosphorus and Cu were measured by spectrophotometer using commercial kits from Biodiagnostic (Diagnostic and Research reagents) and Vitro scient companies, Egypt.

Intestinal histopathology

At the end of experiment (65th week of hen’s age), five hens from each replicate (n= 20/ group) were randomly collected. Birds were killed by cervical dislocation under mild anesthesia. Then, approximately 5 cm of the middle portion of ileum was excised and fixed in 10% formalin for at least 2 days. Slides were prepared according to Bancroft *et al*. (2013), and stained with hematoxylin and eosin (H and E) for hight microscopy.

pH and viscosity of ileal content

An intestinal section from ileum was rinsed with ice-cold physiological saline and opened. Digesta was collected and used for an immediate analysis of dry matter, viscosity and ph. Ileum content viscosity was determined according to method described by Zduńczyk *et al*. (2012).

Statistical analysis

The obtained results were analyzed for analysis of variance (one-way ANOVA) using SAS (2004) to measure the significant differences between the means of different variables. Significance was considered at P < 0.05. Results were presented as mean ± standard error (SE).

RESULTS

Productive performance and egg quality

Replacing the inorganic Cu (CuO) with the organic one (copper polysaccharide complex) at concentrations 4 mg/ kg diet non-significantly increased BW loss of laying hens (P > 0.05). While supplementing Cu from Cu-polysaccharide complex at 8 mg/ kg diet or CuO-NPs (at 4 and 8 mg/ kg diet) significantly increased (P < 0.05) hen’s BW loss compared to the inorganic Cu (Table II). However, using organic Cu and CuO-NPs did not change the FCR and egg mass with a slight increase of egg production (P > 0.05).

Table III shows the effect of different forms of Cu on egg quality. Dietary replacement of CuO with CuO-NPs at 8 mg/ kg diet significantly increased average shell weight (P < 0.05). Whereas, using organic Cu at 4 or 8 mg / kg diet or CuO-NPs at 4 mg/ kg diet did not change shell weight (P > 0.05). Increasing shell weight was associated with a distinct increase of shell thickness in groups supplemented with 4 and 8 mg/ kg diet of CuO-NPs and the higher concentration of organic Cu (P < 0.05). As a result of the weight and thickness increase, the percentage of cracked egg was significantly decreased (P < 0.05). However, replacing the CuO with the organic and Nano forms, except CuO-NPs at 8 mg / kg diet, did not change the internal egg quality in term of yolk weight and index as well as albumen weight and index (P > 0.05). Only, a marked increase of yolk weight was reported in case of replacing CuO with 8 mg/ kg diet of CuO-NPs (P < 0.05). Moreover, organic or CuO-NPs significantly reduced egg yolk cholesterol (P < 0.05). Besides, the lower concentrations of organic and Nano Cu caused a marked higher reduction compared to

Table II. Laying hens’ performance in response to replacement of inorganic copper with organic copper or CuO-NPs.

	Copper source and supplementation levels				
	Inorganic	Organic		Nano	
	8 ppm	8 ppm	4 ppm	8 ppm	4 ppm
55th week	2089.70±20.2	2064.80±22	2090.50±21.2	2073.70±22.8	2081.70±26.3
65th week	2056.70±16.9a	2009.30±24.3b	2054.20±33.6a	2003.60±22.2b	1988.20±27.4b
Body weight changes (65 – 55)	-33.00±4.9c	-55.50±4.7bc	-36.30±14.8c	-70.10±3.4b	-93.50±5a
Feed intake (g/day)	110.00	110.00	110.00	110.00	110.00
Average FCR	2.16±0.04	2.17±0.02	2.16±0.02	2.15±0.02	2.15±0.02
Egg mass	51.19±0.60	50.96±0.50	51.28±0.50	51.26±0.40	51.29±0.30
Average egg production %	78.20±0.60	80.50±0.60	79.60±0.30	83.00±0.3.00	82.50±0.20

Values are means ± SE. Means within the same row with different letters are significantly different at P < 0.05.

Table III. Egg quality in response to dietary replacement of inorganic copper with organic copper or CuO-NPs.

Egg quality parameters	Copper source and supplementation levels				
	Inorganic	Organic		Nano	
	8 ppm	8 ppm	4 ppm	8 ppm	4 ppm
External egg quality					
Average shell weight (g)	6.67±0.19^{b}	6.92±0.23^{ab}	6.71±0.27^{b}	7.30±0.22^{a}	6.87±0.15^{ab}
Average shell thickness (µm)	143.29±4.21^{b}	155.45±4.12^{a}	151.08±5.23^{ab}	159.86±3.34^{a}	152.19±4.23^{ab}
Average cracked shell %	2.20±0.28^{a}	2.10±0.27^{ab}	1.20±0.22^{c}	1.10±0.20^{c}	1.40±0.23^{bc}
Internal egg quality					
Average yolk wt. (g)	17.22±0.55^{ab}	16.53±0.32^{b}	16.99±0.43^{b}	18.65±0.48^{a}	16.93±0.36^{b}
Average yolk Index [1]	0.45±0.03	0.53±0.04	0.45±0.06	0.46±0.05	0.45±0.04
Average albumin wt. (g)	38.75±1.34	40.77±1.12	39.84±1.02	38.93±1.03	39.77±0.96
Average albumin index[2]	8.09±0.22	8.41±0.31	8.36±0.26	8.21±0.33	8.28±0.35
Yolk cholesterol content					
CHO[3] (mg/g)	14.3±0.40^{a}	12.73±0.20^{b}	11.7±0.30b^{c}	12.7±0.28^{b}	11.33±0.30^{c}
(mg/yolk)	246.25±6.89^{a}	213.28±3.09^{b}	218.21±4.34^{b}	236.86±5.99^{a}	191.82±4.89^{c}
Yolk copper content					
Cu (µg/g)	0.32±0.02 ^{b}	0.41±0.01^{a}	0.44±0.02^{a}	0.42±0.02^{a}	0.43±0.03^{a}
Cu (µg/yolk)	5.52±0.34^{b}	6.96±0.19^{a}	7.47±0.32^{a}	7.83±0.36^{a}	7.28±0.45^{a}
Eggshell mineral composition					
Phosphorus%	0.36±0.023	0.46±0.09	0.41±0.05	0.47±0.08	0.40±0.038
Calcium %	33.90±0.8	34.7±1.0	35.26±0.75	34.33±1.4	33.7±0.46

Values are means ± SE. Means within the same row with different letters are significantly different at P < 0.05. [1]Yolk index= Yolk height /Yolk width. [2]Albumen index= Albumen height/ (Albumen length + Albumen width), [3]CHO= cholesterol.

Table IV. Antibody titer production of laying hens in response to replacement of inorganic copper with organic copper or CuO-NPs.

Age/weeks	Copper source and supplementation levels				
	Inorganic	Organic		Nano	
	8 ppm	8 ppm	4 ppm	8 ppm	4 ppm
Antibody titer against avian influenza disease vaccine					
55th	8.2±0.3	8.2±0.2	8.4±0.2	8.2±0.3	8.4±0.2
65th	8.4±0.2^{b}	9.4±0.2^{ab}	9.2±0.5^{ab}	9.4±0.2^{ab}	9.6±0.2^{a}
Antibody titer against new castle disease vaccine					
55th	7.6±0.2	7.4±0.2	7.6±0.2	7.8±0.2	7.4±0.2
65th	8.2±0.3	8.6±0.4	8.8±0.2	9.0±0.0	8.8±0.3
Phagocytic activity	39.99±2.12	40.67±3.26	40.09±4.25	39.56±3.92	41.09±3.25
Phagocytic index	1.88±0.34	1.99±0.09	1.98±0.22	1.86±0.13	2.06±0.13

Values are means ± SE. Means within the same row with different letters are significantly different at P < 0.05.

the others (P < 0.05). On the other hand, replacing CuO with the organic Cu or CuO-NPs significantly increased egg yolk Cu content (P < 0.05) and did not change shell calcium and phosphorus % (P > 0.05).

Immune response

Table IV shows the effect of organic and nano copper replacement on antibody production against avian influenza (AI) disease vaccine or New castle (ND) disease vaccine at the age of 59 and 65 weeks. Neither organic nor CuO-NPs dietary replacement influenced the antibody production in 59 weeks old (P > 0.05). However, on 65th weeks, replacing CuO with organic and CuO-NPs especially, CuO-NPs at 4 mg/ kg diet significantly improved antibody titer against AI vaccine (P < 0.05) while antibody production against ND vaccine was not influenced (P > 0.05). Phagocytic activity and index were not changed because of the dietary replacement of CuO with organic Cu and CuO-NPs (P > 0.05).

Ileum content and blood biochemical constituents

Dietary supplementation of Cu from Cu-polysaccharide complex or CuO-NPs did not alter the pH of ileum content, its moisture and DM % (P >

0.05). Meanwhile, viscosity was significantly decreased following CuO-NPs (at 4 and 8 mg / kg diet) and organic Cu (8 mg / kg diet) supplementation compared with birds receiving inorganic Cu (CuO) (Table V). In Table VI, serum uric acid and creatinine concentrations in addition to liver function related parameters including serum GOT and GPT enzyme activities were non-significantly influenced by organic Cu and CuO-NPs supplementation ($P > 0.05$). On the other hand, blood serum MDA, GPx and SOD activities (Table VI) were significantly changed ($P < 0.05$). Serum GPx activity was distinctly reduced in the organic Cu and CuO-NPs supplemented groups ($P < 0.05$) while MDA and SOD activities were significantly increased ($P < 0.05$). Table VI also demonstrates serum lipid profile including serum triglycerides, total cholesterol, HDL and LDL concentrations following organic Cu and CuO-NPs dietary replacement of CuO. Significant increases were reported for both triglycerides and HDL in case of birds supplemented with organic Cu and CuO-NPs groups ($P < 0.05$). Though, total cholesterol and LDL concentrations were significantly decreased with the organic and Nano source of Cu ($P < 0.05$). Additionally, using organic and Nano sources of Cu in layer's diet instead of the inorganic one did not change the serum calcium and phosphorus contents ($P > 0.05$) but they significantly increased serum Cu level ($P < 0.05$).

Table V. Ileum content character of laying hens in response to replacement of inorganic copper with organic copper or CuO-NPs.

	Copper source and supplementation levels				
	Inorganic	Organic		Nano	
	8ppm	8ppm	4ppm	8ppm	4ppm
pH	6.63±0.03	6.76±0.04	6.98±0.09	6.81±0.8	6.92±0.1
Moisture %	75.8±0.21	74.18±1.6	73.99±0.3	75.49±0.38	74.69±0.26
DM %	24.12±0.2	25.82±1.6	26.0±0.3	24.5±0.3	25.3±0.2
Viscosity	2.25±0.7^{a}	1.99±0.2^{b}	2.15±0.7^{ab}	1.99±0.5^{b}	1.99±0.1^{b}

Values are means ± SE. Means within the same row with different letters are significantly different at $P < 0.05$.

Table VI. Blood serum biochemical parameters and lipid profile of laying hens in response to replacement of inorganic copper with organic copper or CuO-NPs.

Parameters	Copper source and supplementation levels				
	Inorganic	Organic		Nano	
	8 ppm	8 ppm	4 ppm	8 ppm	4 ppm
Kidney function related parameters					
Uric acid (mg/dl)	5.53±0.18^{b}	5.83±0.30^{ab}	5.86±0.80^{ab}	5.90±0.50^{a}	5.90±0.10^{a}
Creatinine (mg/dl)	0.96±0.05	1.03±0.08	0.99±0.060	1.01±0.04	1.03±0.04
Liver function related parameters					
GOT (µ/L)[1]	34.66±1.80	29.66±8.70	30.33±4.30	35.00±1.10	35.00±6.10
GPT (µ/L)[2]	44.00±0.50	43.00±6.50	41.33±3.80	46.00±7.30	51.66±2.70
Antioxidant enzyme activity					
GPx (µ/dl)[3]	367.66±10.3^{a}	338.00±6.50^{b}	315.00±4.50^{c}	313.66±4.20^{c}	308.33±4.40^{c}
MDA (nmol/ml)[4]	9.13±0.28^{b}	11.36±0.80^{a}	10.00±0.36^{ab}	10.73±0.44^{ab}	10.10±0.70^{ab}
SOD[5]	298.23±9.78^{b}	335.67±11.21^{a}	341.21±9.89^{a}	319.09±7.56^{a}	324.23±8.76^{a}
Blood serum lipid profile					
Triglycerides (mg/dl)	195.23±4.1^{b}	198.83±0.3^{ab}	201.2±0.9^{ab}	202.66±0.8^{a}	202.63±1.4^{a}
Total cholesterol (mg/dl)	205.33±2.9^{a}	191.00±4.1^{ab}	186.66±2.00^{b}	179.00±8.1^{b}	185.33±6.8^{b}
HDL (mg/dl)[1]	43.33±2.3^{c}	51.33±1.4^{bc}	55.66±2.4^{ab}	61.33±2^{a}	56.33±5^{ab}
LDL (mg/dl)[2]	122.95±3.9^{a}	99.9±2.7^{b}	90.76±2.5^{bc}	77.13±6^{c}	88.47±6.2^{bc}
Blood serum minerals					
Ca (mg/dl)	19.26±0.30	20.40±0.70	19.50±0.40	19.66±0.10	20.13±0.20
P (mg/dl)	6.03±0.20	5.93±0.10	5.90±0.15	6.03±0.80	5.86±0.10
Cu (µg/dl)	84.00±2.60^{b}	91.00±1.50^{b}	100.00±1.50^{a}	102±2.30^{a}	104.00±2.80^{a}

Values are means ± SE. Means within the same row with different letters are significantly different at $P < 0.05$. [1]Glutamic-oxaloacetic transaminase, [2]glutamic-pyruvic transaminase, [3]glutathione peroxidase, [4]Malondialdehyde, [5]Superoxide dismutase. [6]High density lipoprotein, [6]Low density lipoprotein.

Intestinal histopathology

Laying hens supplemented with inorganic Cu exhibited a normal thickness and villi length of ileum section (Fig. 2). On the other hand, organic Cu supplementation (8mg/ kg diet) reduced the thickness and ileum villi length as well as showed slight necrotic enteritis (Arrow) with moderate lymphoid depletion (Arrowhead), however its supplementation at 4 mg/kg diet improved villi length (VL; Line). Moreover, CuO-NPs at 8 or 4 mg/kg diet instead of inorganic source showed relatively normal intestinal tissues and normal villi.

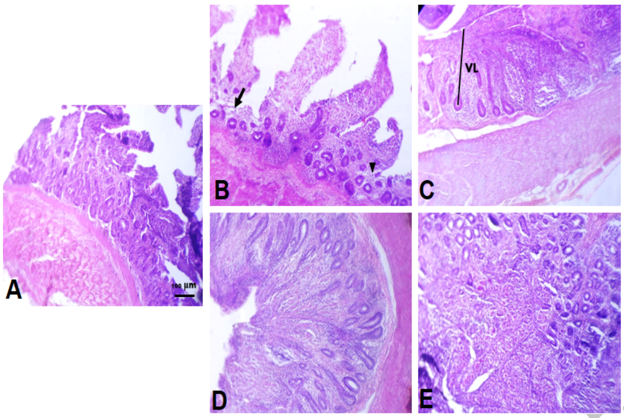

Fig. 2. Ileum of laying hens supplemented with different levels and sources of copper. A, laying hen supplemented with inorganic Cu (8 mg/kg diet), B and C, denote laying hens supplemented with organic Cu (8 and 4 mg/kg diet, respectively), D and E, represent laying hen supplemented with Cu nanoparticles (8 and 4 mg/kg diet, respectively). Arrow represents reduced villi length and thickness as well as slight necrotic enteritis. Arrowhead indicates moderate lymphoid depletion.

DISCUSSION

The present study compared the effect of two different forms of Cu, organic and Nano forms on laying performance of Isa brown hens at the decline phase of egg production compared to its inorganic form (CuO). Micronutrients such as copper are vital elements for layers' performance. They regulate many vital processes in the body including egg production and quality because; they act as co-factors for many enzymes including cytochrome oxidase, tyrosinase, and Cu, Zn-SOD (Abo-Al-Ela *et al.*, 2021). At farm level, the inorganic forms of Cu such as Cu-sulfate and Cu-oxide are supplemented in diets at higher concentrations due to their low bioavailability (El-Kassas *et al.*, 2020). They break down in the gastrointestinal tract forming very reactive free ions that bind to dietary molecules hindering their absorption and consequently decreasing their bioavailability (McDowell, 1992; Close, 1998). Therefore, to get better benefits from the supplemented Cu, it is necessarily to use forms with a higher bioavailability such as organic and Nano Cu. In the current study, we reported that replacing CuO with CuO-NPs or copper polysaccharide complex increased body weight losses at the decline phase of egg production. This might be correlated with the normal expected loss in this phase of production and the slight increase of HDEP reported for hens supplemented with Cu-NPs or copper polysaccharide complex instead of CuO. Moreover, the weight loss might be attributed to the higher concentration of Cu which caused a reduction of intestinal villi length, necrotic enteritis and gastric erosions (Pekel and Alp, 2011). Moreover, the slight increases of HDEP reported in case of organic Cu or CuO-NPs is probably associated with the higher bioavailability and the consequent higher concentration of Cu (Metwally, 2002). Thus, Cu-polysaccharide complex and CuO-NPs could be used safely for aged hens without any effect on laying performance (Maciel *et al.*, 2010). Similar findings were obtained by Sperb *et al.* (1997), and Attia *et al.* (2011) who concluded that egg production was slighted affected by the source and level of Cu. In addition, the different laying responses could be attributed to different Cu bioavailability and consequent different influences within the digestive tract (Pang and Applegate, 2007). On the other side, supplementing Cu from CuO-NPs or copper polysaccharide complex did not change the FCR, and egg mass compared with CuO. These results were similar to Pekel and Alp (2011), and Yenice *et al.* (2015) who reported that organic Cu supplementation had no effect on FCR of laying hens while, disagreed with Idowu *et al.* (2006) who found that organic Cu improved FCR compared with those supplemented by inorganic Cu.

Egg quality, either externally or internally, is influenced by many factors including micro-minerals such as copper (Jegede *et al.*, 2015). The decline phase of egg production, with increasing the hens' age, is characterized by increasing the percentage of cracked egg either because of the depletion of Ca or the other vital elements involved in egg shell formation such as Cu (Park and Sohn, 2018). Previous studies (Attia *et al.*, 2011; Gheisari *et al.*, 2011; Sun *et al.*, 2012; Figueiredo-Júnior *et al.*, 2013) illustrated that using organic trace minerals including Cu did not influence egg quality of laying hens. The current study listed the effect of CuO-NPs (for the first time) and organic Cu on egg quality compared to the inorganic forms. The significant decline of cracked egg percentage was found in case of CuO-NPs might be correlated with a higher bioavailability of CuO-NPs and the increased Cu concentration (El-Kassas *et al.*, 2018; Patra and Lalhriatpuii, 2019). Where, Cu is an important cofactor of the lysyl-oxylase enzyme that helps the formation of collagen cross-links in the egg shell membrane (Chowdhury *et al*., 2004). These results agree with Pang and Applegate (2007), Maciel *et al*. (2010), and Saleh *et al*. (2019) who reported that using organic copper increases Cu concentration helping egg shell membrane formation as well as adjusting the formation of calcium crystals and the holographic structure the eggshell. Additionally, the reduced percentage of cracked egg might be associated with the increased shell's calcium and phosphorus %. This might be related to the slightly higher GIT pH which promoted calcium and phosphorus absorption and consequently improved eggshell quality.

Supplemnting Cu from CuO-NPs or copper polysaccharide complex instead of CuO also, induced a significant reduction of total cholestrol in serum and yolk. The reduced cholestrol might be linked with the increased levels of Cu in serum and yolk, repsectively which reduces the cholestrol synthesis in liver and downregultes the FAS activity (Burkhead and Lutsenko, 2013). The increased Cu level is perhapse associated with an upregulation of Cu-transporters (Ctr1 and ATP7A) which play a major role in the dietary Cu absorption from intestine (Burkhead and Lutsenko, 2013). Moreover, the reduced cholestrol contents might be associated with the reduced glutathione concentration which supports the findings of Pekel and Alp (2011), and Jegede *et al*. (2015). Our findings agreed with Attia *et al*. (2011) and Kim *et al*. (1992) findings who reported that organic Cu reduced egg cholesterol compared with the inorganic source and this reduction was associated with the decrease of serum cholesterol level. Moreover, the less serum cholesterol may be related to a reduced cholesterol synthesis or high degradation and excretion rate leading to an increase of serum HDL (Bakalli *et al*., 1995; Pesti and Bakalli, 1996; Jegede *et al*., 2012).

Copper function in bird's body extends to regulate its immune response through regulating phagocytic activity, controlling host susceptibility to infection, and stimulating the pro-inflammatory response (Goel *et al*., 2013). Previous studies reported that Cu deficiency results in immune alterations manifested by an increased susceptibility to infection with a higher mortality rate (Spears, 2000; Skřivan *et al*., 2002). Although, Jarosz *et al*. (2017) concluded that using organic form of Cu (copper-glycine) stimulated a cellular immune response and encouraged the secretion of cytokines involved in potentiation and regulation of bird's immune response. In the present study, the reported improvement of PA agreed with El-Kassas *et al*. (2018) findings who reported that CuO-NPs significantly increased levels of PA in broiler chickens. Besides, the enhanced effect of Cu supplemented from the organic or Nano sources on the antibody production against ND or AI compared to the CuO was similar to Das *et al*. (2014) findings. These effects are probably due to the higher bioavailability of Cu supplemented from CuO-NPs or copper polysaccharide complex compared to CuO (El-Kassas *et al*., 2018; Patra and Lalhriatpuii, 2019). This improved bioavailability could be explained by the maintenance of GIT pH which is very important for proper function and activity of digestive enzymes, influencing nutrient availability and the balance of gut microbiota keeping the animal healthy (Bristol, 2003). Alteration in pH away from the normal ranges results in reducing digestive and absorptive processes and consequently a significant poor growth performance (Bristol, 2003). Moreover, blood serum MDA and SOD activities were increased while serum GPx activity was reduced in the organic and CuO-NPs supplemented groups. the reported increase of MDA might be associated with an increased level of stress leads to excessive lipid and protein oxidation as well as production of radicals (Ognik and Krauze, 2016). As a result, the antioxidants production inside the body was increased and this was confirmed by the increased level of SOD. Our results suggested that Cu increases the antioxidants activity and enhancing their scavenging function for free radicals. Additionally, we reported a reduction in the antioxidant function of glutathione (GSH) which occurs through the decreased GPx activity. GSH reduces free radicals produced during lipid oxidation as it is oxidized to GSSG which in turn is reduced back to GSH by GSSG reductase (Forman *et al*., 2009). The reduction in the concentration of GPx might be because of its reactions with the oxidants. Also, it is probably linked with increasing the GSSG level which decreases the activity of HMG-CoA reductase (Roitelman and Shechter, 1984) and consequently suppresses the cholesterol synthesis. This effect was confirmed by the low cholesterol level.

CONCLUSION

In conclusion, replacement of copper oxide with organic Cu (Cu polysaccharides complex) or copper nanoparticles in laying hen diet could improve the productive performance, egg quality, and reduce serum or yolk cholesterol. Based on the obtained results, it could be suggested that 4 ppm of organic copper or (8 or 4 ppm /kg diet) of nano source can be used as practical dietary levels for copper supplementation in laying hen diets.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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