Animal Science Journal
Animal Science Journal (2017) ••, ••–•• doi:10.1111/asj.12956

ORIGINAL ARTICLE

The effect of administration of copper nanoparticles to chickens in their drinking water on the immune and antioxidant status of the blood

Katarzyna OGNIK,[1] Iwona SEMBRATOWICZ,[1] Ewelina CHOLEWIŃSKA,[1] Jan JANKOWSKI,[2] Krzysztof KOZŁOWSKI,[2] Jerzy JUŚKIEWICZ[3] and Zenon ZDUŃCZYK[3]

[1]*Department of Biochemistry and Toxicology, Faculty of Biology, Animal Sciences and Bioeconomy, University of Life Science in Lublin, Lublin,* [2]*Department of Poultry Science, Faculty of Animal Bioengineering, University of Warmia and Mazury, and* [3]*Division of Food Science, Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Olsztyn, Poland*

ABSTRACT

The aim of this experiment was to determine what dosage of copper (Cu) nanoparticles, added to a standard dietary supplementation with copper sulfate, would improve antioxidant and immune defense in chickens. The experiment was conducted with 126 broiler chickens assigned to seven treatments with three pens per treatment and six broiler chickens per pen. The basal-diet treatment did not receive Cu nanoparticles (nano-Cu) and as shown by analyses it was Cu-deficient (−29% vs. National Research Council (NRC) recommendations; 5.7 vs. 8 mg/kg). Broiler chickens received nano-Cu (0.5, 1.0 or 1.5 mg/kg body weight per day) via a tube into the crop over three 3-day periods (days 8–10, 22–24 and 36–38) or three 7-day periods (days 8–14, 22–28 and 36–42). As a result, in the nano-Cu-treated broilers the total experimental Cu intake was from −11% to +96% versus NRC recommendations. At the age of 42 days of broilers, their blood indices were determined. The obtained results showed that correction of the deficient basal diet of chickens with nano-Cu to a level of copper exceeding the NRC recommendation by 54% increased the antioxidant potential of the organism and inhibited lipid peroxidation. At the dosage of +96% versus NRC, some symptoms of a deterioration in antioxidant status appeared (a decrease in the level of glutathione plus glutathione disulfide and an increase in superoxide dismutase, catalase and ceruloplasmin activity and in lipid hydroperoxide content). Additionally, +7% versus the recommended Cu dietary level was followed by unfavorable results, indicating a deterioration in red blood cell parameters and stimulation of the immune system (an increase in interleukin-6, immunoglobulin A (IgA), IgM and IgY). To conclude, it was shown that it is possible to simultaneously increase antioxidant and immune defense of chickens by supplementing their diets with nano-Cu – up to 12 mg per bird during 6 weeks of feeding, that is to a level no more than 7% over the NRC recommendation for growing broiler chickens.

Key words: *blood, chicken, immunity, nano-copper, redox status.*

INTRODUCTION

Copper (Cu) is one of the micronutrients considered essential to the growth, development and function of living organisms. Due to its ability to easily accept and donate electrons (it occurs in the oxidation states Cu^{+} and Cu^{2+}), copper is involved in numerous biochemical processes (Angelova *et al.* 2011; Maltais *et al.* 2013); for example, it is part of the active sites of many enzymes, including copper-zinc superoxide dismutase (CuZn-SOD), cytochrome c oxidase, L-lysine oxidase, ascorbate oxidase, tyrosinase and dopamine beta-hydroxylase (Gaetke & Chow 2003). These enzymes play an important role in antioxidant defense, melanin synthesis, formation of connective tissue, dopamine metabolism and mitochondrial respiration (Maltais *et al.* 2013). By inducing conversion of arachidonic acid and synthesis of prostaglandins, Cu takes part in inhibition of inflammation (Angelova *et al.* 2011).

Correspondence: Katarzyna Ognik, Department of Biochemistry and Toxicology, Faculty of Biology, Animal Sciences and Bioeconomy, University of Life Science in Lublin, Lublin, Poland. (Email: kasiaognik@poczta.fm)
Received 28 May 2017; accepted for publication 10 October 2017.

Nevertheless, an excessive concentration of 'free' Cu ions in the cell (the term 'free' copper refers to copper that is not bound to ceruloplasmin) is conducive to free radical damage to proteins, lipids and nucleic acids (Brewer 2007). In the presence of reducing agents, that is ascorbic acid or glutathione (GSH), a Cu^{2+} ion may be reduced to Cu^{+}, which catalyses hydroxyl radical formation from hydrogen peroxide in the Haber-Weiss reaction (Gaetke & Chow 2003; Palumaa 2013; Martínez & Reina 2017). Moreover, Cu can bind directly with thiol groups of sulfur-containing amino acids (cysteine), leading to their oxidation and the formation of crosslinks between proteins, which may result in inactivation of enzymes or damage to the cell's structural proteins (Letelier *et al.* 2005; Wu *et al.* 2010; Dusek *et al.* 2012).

The potential negative effects of an elevated level of Cu in the cell are limited by its binding with intracellular proteins rich in thiol groups (metallothionein and GSH), which exhibit high affinity for Cu ions. Similarly, specialized proteins are involved in intracellular transport of Cu and incorporation of this element into enzyme molecules (Ognik *et al.* 2016).

Due to the physiological functions mentioned above, poultry diets are enriched with Cu, usually by adding copper sulfate (Mikulski *et al.* 2009; Kwiecień *et al.* 2014). In the last decade numerous nutritional experiments have also been carried out using metal nanoparticles, showing that the biological response of the organism depended on the size of the particles, the method by which they were produced, the dosage applied and the length of administration (Zhao & Riediger 2014). It has also been shown, most often *in vitro* using established cell lines (mainly murine macrophages and human dendritic cells) (Małaczewska 2014), that Cu nanoparticles can exert an immunotropic effect, that is react with components of the immune system and thereby stimulate or inhibit it.

The content of Cu in the diet of chickens should be 4 mg/kg in the case of layers and 8 mg/kg for broilers (National Research Council (NRC) 1994). In practice usually more Cu is used. For Ross 308 chickens it is recommended to be 16 mg/kg. The risk of negative consequences of a surplus of Cu in the diet is relatively small (Leeson 2009). Numerous studies, summarized in a review by Leeson (2009), indicate that Cu has toxic effects only when the requirement is exceeded 100 times, while a much smaller, 20-fold increase in the dosage of copper in relation to the nutritional requirement may exert a health-promoting effect by stimulating the immune system. However, it is unknown whether in the case of Cu nanoparticles (nano-Cu), which are potentially better absorbed than Cu from copper sulfate, such a large increase in the level of Cu in the diet is necessary to improve immune and antioxidant defense in chickens.

The aim of the experiment was to determine what dosage of nano-Cu, added to a standard dietary supplementation with copper sulfate, would improve antioxidant and immune defense in chickens.

MATERIALS AND METHODS

Nanoparticles

The subject of the study was an aqueous solution of a copper nanocolloid at a concentration of 50 mg/L. Concentrations of 5, 10 and 15 mg/L were prepared from this solution for the purposes of the experiment. The nano-Cu was non-ionic, nanocrystalline, chemically pure particles 5 nm in size (Fig. 1), produced in a physical process (a non-explosive, high-current method for degradation of metals) by a patented technology licensed by Nano Technologies Group, Inc. (Chicago, IL, USA).

Animals

The material for the study consisted of day-old Ross 308 chickens (♂) raised until the age of 42 days. The experimental procedure was approved by the Second Local Ethics Committee for Experiments with Animals in Lublin (approval no. 30/2014). The birds

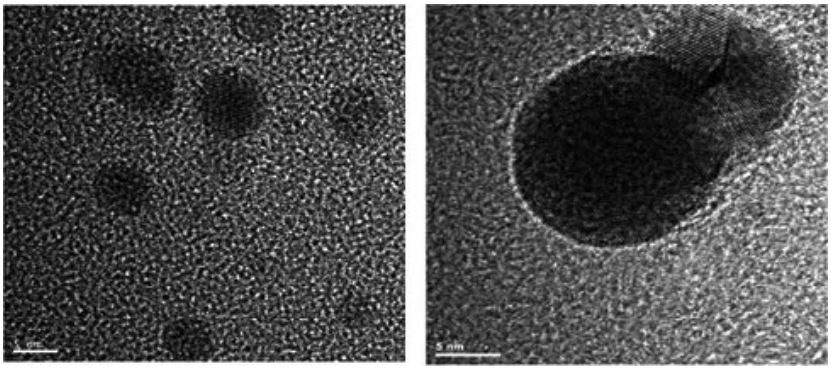

Figure 1 Transmission electron microscopy (TEM) images of cooper nanoparticles (Nano Technologies Group, Inc.).

were kept in pens on straw litter and reared in standard conditions in a building with regulated temperature and humidity. They had permanent access to drinking water and received ad libitum complete feed mixtures appropriate for the rearing period in accordance with feeding standards for poultry (NRC 1994) (Table 1). The experiment was carried out on 126 chicks assigned to seven experimental groups of 18 birds each (three replications of six individuals each).

The experimental design is shown in Table 2. The basal-diet group (BN) did not receive nano-Cu. The chickens in groups $T1_{0.5}$ and $T2_{0.5}$ received nano-Cu at a dose of 0.5 mg/kg body weight (BW)/day, groups $T1_{1.0}$ and $T2_{1.0}$ received 1.0 mg/kg BW/day, and groups $T1_{1.5}$ and $T2_{1.5}$ were given 1.5 mg/kg BW/day. The chickens received nano-Cu via a tube into the crop: groups $T1_{0.5}$, $T1_{1.0}$ and $T1_{1.5}$ in three 3-day cycles (8–10, 22–24 and 36–38 days of life) and groups $T2_{0.5}$, $T2_{1.0}$ and $T2_{1.5}$ in three 7-day cycles (8–14, 22–28 and 36–42 days of life). In establishing the periods during which the nano-Cu would be administered we took into account the fact that application during the entire rearing period would not be feasible due to their high cost. There are no studies in the available literature on the effect of the duration of application of nano-Cu in terms of accumulation of this element and its toxic effects on chickens. The choice of the weeks and duration of the administration was experimental. The chickens received a balanced compound feed in which the Cu content was determined (Table 1). Taking into account the standards recommended by the NRC (1994) we calculated the percentage of the requirement for Cu received by the chickens (Table 2).

Table 1 Composition of basal non-supplemented diets for broiler chickens

Ingredients (g/kg)	Starter weeks 1–3	Grower weeks 4–5	Finisher week 6
Wheat	452.8	367.6	330.7
Maize	150.0	250.0	300.0
Soybean meal (46% protein)	272.2	227.9	178.1
Rapeseed meal (37% protein)	20.0	40.0	60.0
Soybean oil	20.0	40.0	60.0
DDGS† (26% protein)	40.07	43.58	46.87
Monocalcium phosphate	11.03	5.42	2.05
$CaCO_3$	16.07	10.93	8.52
NaCl	3.63	3.23	2.83
DL-Met (99%)	3.61	2.40	2.00
L-Lys HCl	4.27	2.97	3.12
L-Thr (99%)	1.31	0.94	0.82
Premix‡,§	5.0	5.0	5.0
Calculated composition			
Metabolizible energy (kcal/kg)	3070	3140	3190
Crude protein (g/kg)	210.0	198.5	187.5
Crude fiber (g/kg)	27.2	29.8	32.2
Crude fat (g/kg)	65.9	74.5	81.4
Lys (g/kg)	13.5	11.7	10.9
Met (g/kg)	6.7	5.5	5.0
Met + Cys (g/kg)	10.1	8.8	8.3
Trp (g/kg)	2.5	2.3	2.1
Arg (g/kg)	13.1	12.1	11.1
Ca (g/kg)	9.8	7.3	6.0
P available (g/kg)	3.9	2.8	2.1
Na (g/kg)	1.6	1.5	1.4
Analyzed minerals composition			
Fe (mg/kg)	67.2	49.3	57.6
Ca (g/kg)	9.74	7.33	6.12
Cu (mg/kg	5.61	5.76	5.69

†DDGS – maize distillers dried grains with solubles. ‡Vitamin provided per kilogram of diet. Weeks 1–3: vitamin A, 15 000 IU; vitamin D3, 5000 IU; vitamin E. 112 IU; vitamin K_3, 4 mg; vitamin B_1, 3 mg; vitamin B_2, 8 mg; vitamin B_6, 5 mg; vitamin B_{12}, 16 mg; folic acid, 2 mg; biotin, 0.2 mg; nicotinic amid, 60 mg; calcium pantothenicum, 18 mg; choline, 1.8 g. Weeks 4–5: vitamin A, 12 000 IU; vitamin D_3, 5000 IU; vitamin E, 75 IU; vitamin K_3, 2 mg; vitamin B_1, 2 mg; vitamin B_2, 6 mg; vitamin B_6, 4 mg; vitamin B_{12}, 16 mg; folic acid, 1.75 mg; biotin, 0.05 mg; nicotinic amid, 60 mg; calcium pantothenicum, 18 mg; choline, 1.6 g. Week 6: vitamin A, 12 000 IU; vitamin D_3, 5000 IU; vitamin E, 75 IU; vitamin K_3, 2 mg; vitamin B_1, 2 mg; vitamin B_2, 5 mg; vitamin B_6, 3 mg; vitamin B_{12}, 11 mg; folic acid, 1.5 mg; biotin, 0.05 mg; nicotinic amid, 35 mg; calcium pantothenicum, 18 mg; choline, 1.6 g. §Trace minerals provided per kilogram of diet (declared by the manufacturer): Mn, 100 mg; Zn, 80 mg; Fe, 80 mg; Cu in the form of copper sulfate, 8 mg; I, 1 mg; Se, 0.15 mg; coccidiostat – salinomycin (except week 6); please see analyzed content of Fe and Cu.

Laboratory analysis

Blood for analysis was collected into test tubes with an anticoagulant (heparin) from the wing vein of all chickens at the age of 42 days. Next the blood samples were centrifuged at 3000 × *g* for 10 min and the plasma was collected for further analysis.

Hemoglobin content (Hb), hematocrit (Ht), erythrocyte count (RBC) and leukocyte count (WBC) in the blood were determined in an Abacus Junior Vet hematology analyzer (Diatron, Budapest, Hungary). The Wintrobe method was used to determine the erythrocyte sedimentation rate (ESR) in the blood, that is the rate at which erythrocytes settle out of unclotted blood in 1 h (Bomski 1995). Ceruloplasmin in the blood plasma (Cp) was determined by the p-phenylenediamine colorimetric method according to Sunderman and Nomoto (1970). Immunoglobulins IgA, IgM and IgY and interleukin (IL)-6 in the blood were determined in an ELISA reader using assays from Elabscience Biotechnology Co., Ltd. (Houston, Texas, USA). Serum lysozyme activity was determined by the turbidimetric method (Siwicki & Anderson 1993). SOD in the erythrocytes was determined using a Ransod diagnostics kit from Randox (London, UK) and catalase activity (CAT) was determined according to Aebi (1984). Plasma content of GSH content (+ glutathione disulfide (GSSG)), lipid

Table 2 Experimental design and doses of nano-Cu administered to chickens

Item	Treatment						
	BN	$T1_{0.5}$	$T1_{1.0}$	$T1_{1.5}$	$T2_{0.5}$	$T2_{1.0}$	$T2_{1.5}$
Daily dose of nano-Cu hydrocolloids (mL)							
Week 2	0	41	41	41	41	41	41
Week 4	0	128	128	128	128	128	128
Week 6	0	235	235	235	235	235	235
Concentration of nano-Cu (mg/L)	0	5	10	15	5	10	15
Cyclical administration of nano-Cu†	0	3 × 3	3 × 3	3 × 3	3 × 7	3 × 7	3 × 7
Total nano-Cu applied (mg/bird)	0	6.06	12.12	18.21	14.14	28.28	42.48
Total intake of Cu‡ (mg/bird)	24.22	30.28	36.34	42.43	38.36	52.50	66.70
Cu intake in relation to NRC (1994) recommendation (%)§	−28.7	−11	+7	+25	+13	+54	+96

†3 × 3 – administration on days 8–10, 22–24 and 36–38 of life or 3 × 7 – administration on days 8–14, 22–28 and 36–42 days of life. ‡In group C intake only in feed on days 1–42 days of life, in other groups total Cu intake in feed and nano-Cu hydrocolloids. §In accordance with the national Research Council (NRC) recommendation (1994) the reference point would be a diet containing 8 mg Cu/kg and average consumption of 4.25 kg fodder during days 1–42 days of life. BN, group fed basal non-supplemented with additional Cu diet; T1, received Cu nanoparticles via a tube into the crop in three 3-day periods (days 8–10, 22–24 and 36–38); T2, received Cu nanoparticles via a tube into the crop in three 7-day periods (days 8–14, 22–28 and 36–42).

hydroperoxides (LOOH) and malondialdehyde (MDA), as well as total antioxidant potential (FRAP), were determined according to methods described by Ognik and Wertelecki (2012).

Statistical analysis

The model assumptions of normality and homogeneity of variance were examined by the Shapiro–Wilk and Levene tests, respectively. To compare the BN group (untreated with nano-Cu) versus each experimental group (treated with nano-Cu), the data were subjected to a Student's *t*-test procedure. In a model without the untreated group (BN), two-way analysis of variance was performed to examine the main effects: D – nano-Cu dose effect (0.5, 1.0 and 1.5 mg/kg BW per day), T – time effect (two variants of cyclical administration of nano-Cu; three cycles × 3 days and three cycles × 7 days; T1 and T2, respectively), and the interaction between these two factors (D × T). If the analysis revealed a significant interaction ($P \leq 0.05$), the differences between treatment groups ($T1_{0.5}$, $T1_{1.0}$, $T1_{1.5}$, $T2_{0.5}$, $T2_{1.0}$ and $T2_{1.5}$) were then determined with the Newman–Keuls post hoc test at $P \leq 0.05$. The statistical analysis was performed according to the GLM procedure for Statistica 8.0PL software (StatSoft Corp., Krakow, Poland). Treatment effects were considered to be significant at $P \leq 0.05$. All data were expressed as mean values with pooled SE. A Student's *t*-test was used to compare the slopes of the regression equations.

RESULTS

Nano-Cu administered to chickens had no effect on the growth performance parameters of the chickens (body weight gain in 1–42 days of age was 2.32–2.37 kg per bird, feed conversion in 1–42 days of age was 1.74–1.76 kg/kg weight gain).

The experimental treatments, that is cyclical per os administration of a hydrocolloid of nano-Cu, and the mean daily intake of Cu by the chickens are presented in Table 2. In treatment $T1_{0.5}$ the addition of nanoparticles reduced the Cu deficiency from 28.7% to 11% in relation to the level recommended by the NRC (1994). In the remaining treatments, the mean daily intake of Cu in the diet and in the nanoparticles, the supply of this element exceeded the recommended amount by 7% ($T1_{1.0}$) to nearly 100% ($T2_{1.5}$).

Effect of the dosage of nano-Cu

As the concentration of Cu increased in the hydrocolloid of nano-Cu, the RBC count increased ($P = 0.05$) while WBC and ESR decreased ($P = 0.041$ and $P = 0.047$, respectively) in the blood of the chickens (Table 3). Increasing the dosage of nano-Cu resulted in an increase in the level of immunoglobulins, that is IgA ($P = 0.01$), IgM ($P = 0.05$) and IgY ($P = 0.014$), and the cytokine IL-6 ($P = 0.018$) in the blood of the chickens, and a decrease in lysozyme activity ($P = 0.032$) (Table 4). Increasing the dosage of nano-Cu resulted in an increase in the activity of SOD, CAT and Cp ($P = 0.028$, $P = 0.019$ and $P = 0.048$, respectively) (Table 5), and in the content of LOOH ($P = 0.05$) and MDA ($P = 0.036$) in the blood plasma (Table 6).

Effect of the time of administration of nano-Cu

The longer period of administration of nano-Cu to the chickens (T2) led to a decrease in Hb, Ht and

Table 3 Hematological indices of the chickens

	Hb (L/L)	Ht (L/L)	RBC (10^{12}/L)	WBC (10^9/L)	ESR (mm/h)
BN (n = 6)	14.3	26.2	2.32	25.6	3.62
Nano-Cu treated (n = 6)					
$T1_{0.5}$	15.02[a]*	26.94	2.495*	24.32*	3.50
$T1_{1.0}$	15.12[a]*	25.65	2.556*	23.14*	3.16**
$T1_{1.5}$	14.82[ab]*	25.43*	2.991*	23.33*	2.75*
$T2_{0.5}$	14.06[c]	24.92*	2.272	25.43	3.51
$T2_{1.0}$	14.63[b]	24.33*	2.296	24.69	3.52
$T2_{1.5}$	14.21[bc]	24.87*	2.314	24.48	3.38
SEM	0.123	0.264	0.178	0.088	0.093
Dosage effect (D)					
0.5	14.5	25.9	2.38[b]	24.9[a]	3.50[a]
1.0	14.9	25.0	2.43[b]	24.0[ab]	3.34[b]
1.5	14.5	25.1	2.65[a]	23.9[b]	3.06[c]
Time effect (T)					
T1	15.0	26.0	2.68	23.6	3.05
T2	14.3	24.7	2.29	24.9	3.47
P-value					
D effect	0.088	0.062	0.050	0.041	0.047
T effect	0.050	0.033	0.031	0.035	0.034
D × T interaction	0.034	0.061	0.142	0.070	0.089

*Means within the same column differ significantly from the BN at $P \leq 0.05$ according to Student's t-test procedure. [a–c]Means within the same column differ significantly ($P \leq 0.05$) according to Newman–Keuls mean comparison (only in the case of significant D×T interaction). SEM, standard error of the mean (SD for all chickens divided by square root of number of chickens, n = 40); Hb, hemoglobin; Ht, hematocrit; RBC, red blood cells (erythrocytes); WBC, white blood cells (leukocytes); ESR, erythrocyte sedimentation rate; BN, group fed basal non-supplemented with additional Cu diet; T1, received Cu nanoparticles via a tube into the crop in three 3-day periods (days 8–10, 22–24 and 36–38); T2, received Cu nanoparticles via a tube into the crop in three 7-day periods (days 8–14, 22–28 and 36–42).

RBC (P = 0.05 and P = 0.033 and P = 0.031, respectively) and an increase in WBC and ESR (P = 0.025 and P = 0.034) in the plasma (Table 3). In the case of Hb content, a D × T interaction was observed, as the value for this parameter was higher in the $T1_{0.5}$ and $T1_{1.0}$ treatments, there was no difference between treatments $T1_{1.5}$, $T2_{1.0}$ and $T2_{1.5}$, and the lowest Hb content was noted for the $T2_{0.5}$ treatment (Table 3).

As compared with the T1 treatments, the chickens from the T2 treatments had a higher level of IgA (P = 0.032), IgM (P = 0.047) and IgY (P = 0.022) and of the cytokine IL-6 (P = 0.043) in the blood plasma (Table 4). In the chickens from the T2 treatments lower lysozyme activity was also observed in the blood plasma (P = 0.047). Moreover, a statistical interaction of D × T was noted in the case of IgA, IgM and IgY, as the plasma concentration of these immunoglobulins increased in the case of the highest dosage of nano-Cu administered for the longer time (T2).

The longer period (T2) of administration of nano-Cu caused an increase in CAT activity ($P < 0.001$) and a decrease in that of Cp ($P < 0.001$). A D × T interaction was also observed for CAT and Cp (P = 0.044 and P = 0.05) (Table 5). In the chickens from the T2 treatments an increased plasma concentration of LOOH (P = 0.047) was observed (Table 6).

Effect of total intake of copper: Cu_2SO_4 and nano-Cu

In the $T1_{0.5}$ and $T1_{1.0}$ treatments, in which the addition of nano-Cu reduced the Cu deficiency from 29% to 11% or increased the supply of this element by 7% in relation to the level recommended by the NRC (1994), an increase in RBC count and a decrease in WBC count and ESR were expressed by the simple regression equations, with very high coefficients of determination: R^2 = 0.926, R^2 = 0.999 and R^2 = 0.952, respectively (Table 7). In the treatments in which the addition of nano-Cu increased the intake of Cu to above NRC recommendations (1994), linear decreases in WBC, Hb, Ht and lysozyme were observed in the blood of the chickens, described by highly significant regression equations (R^2 = 0.905, R^2 = 0.979, R^2 = 0.992 and R^2 = 0.919, respectively).

Similar relationships were noted in the case of the immune and antioxidant parameters of the chicken blood. In the $T1_{0.5}$ and $T1_{1.0}$ treatments a linear increase was noted for IL-6 (R^2 = 0.987) and CAT (R^2 = 0.926), while in the $T1_{1.5}$, $T2_{0.5}$, $T2_{1.0}$ and $T2_{1.5}$ treatments a linear increase was observed for the level of IgA, IgM, IgY and LOOH, described by highly significant regression equations (R^2 = 0.997, R^2 = 0.994, R^2 = 0.997 and R^2 = 0.870, respectively). Moreover, in the treatments which increased the Cu

Table 4 Immune parameters in the blood of the chickens

Item	IgA (ng/mL)	IgM (ng/mL)	IgY (ng/mL)	IL-6 (pg/mL)	Lysozyme (μg/L)
BN (n = 6)	20.93	191.0	630.0	6.253	3.121
Nano-Cu treated (n = 6)					
$T1_{0.5}$	21.11^{d}	171.0^{d}	630.2^{b}	6.032*	3.098
$T1_{1.0}$	22.52^{c}	226.0^{c}	636.3^{c}	6.781*	3.114
$T1_{1.5}$	32.16^{b}*	277.2^{b}*	653.0^{b}*	6.522*	3.124
$T2_{0.5}$	25.12^{c}*	288.0^{ab}*	632.4^{c}	6.454*	3.642*
$T2_{1.0}$	45.42^{a}*	311.0^{a}*	625.0^{c}	6.982*	3.044
$T2_{1.5}$	45.68^{a}*	314.3^{a}*	728.1^{a}*	9.821*	1.892*
SEM	0.145	0.278	0.092	0.463	0.721
Dosage effect (D)					
0.5	23.11^{b}	229.0^{b}	631.3^{b}	6.243^{c}	3.370^{a}
1.0	33.97^{a}	268.0^{a}	630.6^{b}	6.881^{b}	3.079^{b}
1.5	38.92^{a}	295.7^{a}	690.5^{a}	8.171^{a}	2.508^{c}
Time effect (T)					
T1	25.26	224.7	639.8	6.445	3.112
T2	38.74	304.7	661.5	7.752	2.859
P-value					
D effect	0.010	0.050	0.014	0.018	0.032
T effect	0.032	0.047	0.022	0.043	0.047
D × T interaction	0.050	0.033	0.013	0.171	0.149

*Means within the same column differ significantly from the BN at $P \leq 0.05$ according to Student's t-test procedure. Data from groups treated with nano-Cu subjected to two-way analysis of variance. $^{a-d}$Means within the same column differ significantly ($P \leq 0.05$) according to Newman–Keuls mean comparison (only in the case of significant D × T interaction). SEM, standard error of the mean (SD for all chickens divided by square root of number of chickens, n = 40); IgA, immunoglobulin A; IgM, immunoglobulin M; IgY, immunoglobulin Y; IL-6, interleukin 6; BN, group fed basal non-supplemented with additional Cu diet; T1, received Cu nanoparticles via a tube into the crop in three 3-day periods (days 8–10, 22–24 and 36–38); T2, received Cu nanoparticles via a tube into the crop in three 7-day periods (days 8–14, 22–28 and 36–42).

supply to above NRC recommendations (1994), a linear increase was also noted in Cp activity in the blood plasma (R^2 = 0.999).

DISCUSSION

Numerous studies have shown that Cu plays a significant role in iron metabolism, hemoglobin synthesis, and erythrocyte production (Tapiero *et al.* 2003; Sharma *et al.* 2009; Samanta *et al.* 2011), as Cp, which transports about 95% of Cu contained in blood, also takes part in iron metabolism (Meyer *et al.* 2001; Zerounian & Linder 2002; Cherukuri *et al.* 2004). As a component of blood, iron takes part in the transport of oxygen by hemoglobin. Cp, by oxidizing Fe^{2+} to Fe^{3+}, enables binding of iron to transferrin, which is a fundamental process for the transport of iron from the bloodstream to the cells, mediated by the transferrin receptor (Tapiero *et al.* 2003). For this reason changes in hematological parameters have been noted in experiments on poultry using nano-Cu (Mroczek-Sosnowska *et al.* 2013; Ghasemipoor & Zolghadri 2014; Miroshnikov *et al.* 2015).

An increase in erythrocyte parameters (increased RBC, Hb and Ht and decreased WBC) was observed in the blood of chickens following in ovo injection of 0.3 mL of a hydrocolloid of 37 nm nano-Cu at a concentration of 50 mg/kg into the air chamber of fertilized eggs of broiler chickens (Mroczek-Sosnowska *et al.* 2013). In another experiment (Miroshnikov *et al.* 2015), increased RBC and Hb were noted in the blood of chickens following intramuscular administration of nano-Cu or microparticles (nanoparticles – 103 ± 2 nm, nanoparticle agglomerates – 937 ± 24.6 nm and microparticles 40 ± 0.5 um). The authors also found that the rate of erythropoiesis was dependent on the size of the Cu particle. Increased content of RBC and Hb was not noted until the 7th day after injection of nanoparticle agglomerates, but was observed just 1 day after injection of nano-Cu; moreover, only the effect of nano-Cu was long-lasting. Different results, that is a reduction in hemoglobin content in the blood, were noted in chickens receiving nano-Cu at a dose of 16 mg/kg BW for a period of 35 days (Ghasemipoor & Zolghadri 2014). In the present study, the level of the hematological parameters depended on the dosage of nano-Cu. In the treatments in which the Cu content was below NRC (1994) recommendations, the addition of nano-Cu resulted in an increase in Cp activity as well as in hemoglobin content, hematocrit and erythrocyte count, and a decrease in the leukocyte count and the ESR. A higher level of dietary supplementation with nano-Cu, raising the amount of Cu to 13% or more over the NRC (1994) recommendation, resulted in a decrease in WBC and ESR, but also in Hb, Ht and

Table 5 Activity of antioxidant enzymes SOD, CAT and Cp in the blood of chickens

Group	SOD (U g Hb)	CAT (U g Hb)	Cp (U/L)
BN (n = 6)	863.8	435.8	0.261
Nano-Cu treated (n = 6)			
$T1_{0.5}$	851.9	419.6^{b}	0.333^{c}*
$T1_{1.0}$	853.9	482.3^{ab}*	0.690^{a}*
$T1_{1.5}$	856.7	458.9^{ab}	0.730^{a}*
$T2_{0.5}$	855.8	469.5^{ab}	0.292^{d}*
$T2_{1.0}$	854.4	458.7^{ab}	0.370^{c}*
$T2_{1.5}$	924.8*	503.6^{a}*	0.450^{b}*
SEM	0.088	0.163	0.551
Dosage effect (D)			
0.5	853.8^{b}	444.5^{b}	0.312^{c}
1.0	854.1^{b}	470.5^{a}	0.530^{b}
1.5	890.7^{a}	481.2^{a}	0.590^{a}
Time effect (T)			
T1	854.1	320.2	0.584
T2	878.3	477.2	0.370
P-value			
D effect	0.028	0.019	0.048
T effect	0.745	<0.001	<0.001
D × T interaction	0.216	0.044	0.050

*Means within the same column differ significantly from the BN at $P \leq 0.05$ according to Student's t-test procedure. Data from groups treated with nano-Cu subjected to two-way analysis of variance. [a–d]Means within the same column differ significantly ($P \leq 0.05$) according to Newman–Keuls mean comparison (only in the case of significant D×T interaction). SEM, standard error of the mean (SD for all chickens divided by square root of number of chickens, n = 40; SOD, superoxide dismutase; CAT, catalase; Cp, ceruloplasmin; BN, group fed basal non-supplemented with additional Cu diet; T1, received Cu nanoparticles via a tube into the crop in three 3-day periods (days 8–10, 22–24 and 36–38); T2, received Cu nanoparticles via a tube into the crop in three 7-day periods (days 8–14, 22–28 and 36–42).

RBC. However, the hematology values were within the normal (physiological) range for chickens (Mitruka & Rawnsley 1977; Abdulazeez *et al.* 2016).

The lowest dose of nano-Cu (0.5 mg/kg BW/day) administered to the chickens for the shorter time period (T1) reduced the level of the pro-inflammatory cytokine IL-6. This was the only treatment in which supplementation of the diet of the chickens with nano-Cu, which reduced the Cu deficit from 28.7% to 11% in relation to NRC (1994) recommendations, did not stimulate an inflammatory response, and thus was well tolerated by the organism. In all other treatments, which increased the supply of Cu in the diet to above NRC (1994) recommendations, an increase was noted in the level of IL-6 in the blood of the chickens. This was consistent with the simultaneous increase in Cp activity in the blood of the chickens following these treatments.

Ceruloplasmin, apart from its role in erythropoiesis, is included among positive acute phase proteins. Its production increases as a result of infection and inflammation, which is probably linked to the response of the organism to oxidative stress accompanying these processes (Salih 2010). Sui *et al.* (2011) administered copper oxide nanoparticles to chickens in their feed at 8 or 175 mg/kg and noted a significant increase in Cp activity in the blood. In our experiment, the longer period (T2) of administration of nano-Cu led to inhibition of Cp activity, but caused an increase in other inflammatory markers, that is ESR and IL-6. IL-6, which is sometimes described as a growth factor for B lymphocytes, is considered the most important cytokine responsible for activation of B cells (Dinant & Dijkmans 1999). In the inflammatory reaction there is an increase in the concentration of pro-inflammatory cytokines (including IL-6 and ILs stimulating synthesis of acute phase proteins, i.e. C-reactive protein and Cp), which are responsible for restoring homeostasis (Polińska *et al.* 2009). The changes taking place in the proportions of individual serum proteins during inflammation (an increase in globulins and fibrinogen and a decrease in albumins) result in a faster ESR.

Our study showed that as the dosage of nano-Cu increased, particularly where the level of Cu in the diet exceeded NRC (1994) recommendations by more than 7%, the content of immunoglobulins (IgA, IgM and IgY) increased while lysozyme activity decreased in the blood of the chickens. This is consistent with results reported by Wang *et al.* (2011), who found that administration of nano-Cu to chickens with chitosan in the amounts of 50, 100, 150 mg/kg of feed caused an increase in the level of IgA, IgM and IgG as well as complements C3 and C4 in the blood. Another experiment (Pineda *et al.* 2013), in which a hydrocolloid of nano-Cu was injected into the air chamber of the fertilized eggs of broiler chickens, revealed no effect on the levels of IgG or IgM or on expression of messenger RNA, nuclear factor-κ8 or tumor necrosis factor in the blood of the chickens.

The increase in the content of immunoglobulins in our experiment may indicate stimulation of B lymphocytes resulting from direct interaction with the nano-Cu or indirectly induced by cytokines released from macrophages or other phagocytes. Because the changes in the concentrations of immunoglobulins were correlated with an increase in the concentration of IL-6, the increase in immunoglobulin may have been the result of stimulation of phagocytes. While this question requires more in-depth analysis, the increased concentration of IgA in the blood of the chickens should be considered a cause for concern, as it may indicate an autoimmune reaction.

Many studies, summarized in a review by Leeson (2009), indicate that moderately high levels of dietary Cu seem to affect lipid metabolism, for example by reducing cholesterol levels in poultry products.

Table 6 Antioxidant parameters of the blood of the chickens

Group	LOOH (μmol/L)	MDA (μmol/L)	FRAP (μmol/L)	GSH + GSSG (μmol/L)
BN (n = 6)	21.18	1.239	138.3	0.253
Nano-Cu treated (n = 6)				
$T1_{0.5}$	18.33*	1.164*	142.5*	0.267
$T1_{1.0}$	18.24*	1.153*	152.2*	0.268
$T1_{1.5}$	18.27*	1.240	135.9	0.261
$T2_{0.5}$	18.69	1.187*	125.3*	0.257
$T2_{1.0}$	19.36	1.182*	143.8*	0.264
$T2_{1.5}$	23.15	1.177*	148.6*	0.174*
SEM	0.139	0.238	0.381	0.033
Dosage effect (D)				
0.5	18.51[b]	1.175[b]	133.9	0.262
1.0	18.80[b]	1.167[b]	148.0	0.266
1.5	20.71[a]	1.208[a]	142.2	0.217
Time effect (T)				
T1	18.28	1.185	143.5	0.265
T2	20.40	1.182	139.2	0.231
P-value				
D effect	0.050	0.036	0.245	0.146
T effect	0.047	0.147	0.142	0.214
D × T interaction	0.098	0.254	0.264	0.145

*Means within the same column differ significantly from the BN at $P \leq 0.05$ according to Student's t-test procedure. Data from groups treated with nano-Cu subjected to two-way analysis of variance. SEM, standard error of the mean (SD for all chickens divided by square root of number of chickens, n = 40); LOOH, lipid hydroperoxides; MDA, malondialdehyde; FRAP, total antioxidant status; GSH + GSSG, total glutathione; BN, group fed basal non-supplemented with additional Cu diet; T1, received Cu nanoparticles via a tube into the crop in three 3-day periods (days 8–10, 22–24 and 36–38); T2, received Cu nanoparticles via a tube into the crop in three 7-day periods (days 8–14, 22–28 and 36–42).

Table 7 Effect of total copper intake on linearity of changes in selected blood parameters

Item	Regression equation	Comment
Hb (L/L)	$y = -0.0284x + 16.102$; $R^2 = 0.979$	Linear decrease in total Cu intake in the range of 36.34–66.70 mg
Ht (L/L)	$y = -0.0249x + 26.52$; $R^2 = 0.992$	Linear decrease in total Cu intake in the range of 36.34–66.70 mg
RBC (10^{12}/L)	$y = 0.0197x + 1.8589$; $R^2 = 0.926$	Linear increase in total Cu intake in the range of 24.22–36.34 mg
WBC (10^9/L)	$y = -0.2054x + 30.584$; $R^2 = 0.999$	Linear decrease in total Cu intake in the range of 24.22–36.34 mg
	$y = -0.0335x + 26.627$; $R^2 = 0.905$	Linear decrease in total Cu intake in the range of 38.36–66.70 mg
ESR (mm/h)	$y = -0.0486x + 4.8773$; $R^2 = 0.952$	Linear decrease in total Cu intake in the range of 24.22–42.43 mg
IgA (ng/mL)	$y = 1.4241x - 29.087$; $R^2 = 0.997$	Linear increase in total Cu intake in the range of 36.34–52.50 mg
IgM (ng/mL)	$y = 6.2727x - 7.0399$; $R^2 = 0.944$	Linear increase in total Cu intake in the range of 30.28–52.50 mg
IgY (ng/mL)	$y = 2.8869x - 532.38$; $R^2 = 0.965$	Linear increase in total Cu intake in the range of 30.28–66.70 mg
IL-6 (pg/mL)	$y = 0.0418x - 4.7874$; $R^2 = 0.987$	Linear increase in total Cu intake in the range of 36.34–52.50 mg
Lizosyme (μg/L)	$y = -0.0561x + 5.7297$; $R^2 = 0.919$	Linear decrease in total Cu intake in the range of 42.43–66.70 mg
SOD (U g Hb)	–	No linearity
CAT (U g Hb)	$y = 2.273x + 352.4$; $R^2 = 0.926$	Linear increase in total Cu intake in the range of 30.28–66.70 mg
Cp (U/L)	$y = 0.0354x - 0.6438$; $R^2 = 0.892$	Linear increase in total Cu intake in the range of 24.22–36.34 mg
	y = 0.0056x+0.0779; $R^2 = 0.999$	Linear increase in total Cu intake in the range of 38.36–66.70 mg
LOOH (μmol/L)	$y = 0.1575x + 12.131$; $R^2 = 0.870$	Linear increase in total Cu intake in the range of 38.36–66.70 mg
MDA (μmol/L)	$y = -0.0004x + 1.2005$; $R^2 = 0.998$	Linear decrease in total Cu intake in the range of 38.36–66.70 mg
FRAP (μmol/L)	$y = 1.1469x + 109.61$; $R^2 = 0.950$	Linear increase in total Cu intake in the range of 24.22–36.34 mg
	$y = 0.7433x + 101.24$; $R^2 = 0.850$	Linear increase in total Cu intake in the range of 42.43–66.70 mg
GSH + GSSG (μmol/L)	–	No linearity

Hb, hemoglobin; Ht, hematocrit; RBC, red blood cells (erythrocytes); WBC, white blood cells (leukocytes); ESR, erythrocyte sedimentation rate; IgA, immunoglobulin A; IgM, immunoglobulin M; IgY, immunoglobulin Y; IL-6, interleukin 6; SOD, superoxide dismutase; CAT, catalase; Cp, ceruloplasmin; LOOH, lipid hydroperoxides; MDA, malondialdehyde; FRAP, total antioxidant status; GSH + GSSG, total glutathione

Another effect of dietary Cu may be antioxidant protection (Song *et al.* 2009; Sui *et al.* 2011; Pineda *et al.* 2013). According to Pineda *et al.* (2013), by injecting 0.3 mL of a hydrocolloid of nano-Cu (2–15 nm, 50 mg/kg) into the air chamber of a fertilized egg, it is possible to reduce the intensity of oxidative

processes in growing chickens. The authors of the study found that chicks hatched from eggs subjected to this procedure, irrespective of the day of injection of nano-Cu, had a significantly lower metabolic rate, which was linked to lower oxygen consumption. These results suggest that lower oxygen consumption in the body reduces generation of free oxygen radicals and at the same time reduces oxidation of important cell structures.

Our study showed that the treatments raising the level of Cu in the diet closer to that recommended by the NRC (1994) or to a level exceeding the recommendation, particularly by 7%, caused an increase in the FRAP value in the blood plasma. Moreover, a level of dietary Cu exceeding the recommended level by 7% or more increased CAT and Cp activity and decreased (except for the largest dosage of nano-Cu) the plasma level of LOOH. The reduced content of MDA in the blood of birds from all treatments indicates that the addition of nano-Cu did not increase lipid peroxidation in the chickens. Only in the treatment in which the level of Cu recommended by the NRC (1994) was exceeded by 96% was an increase noted in SOD activity and a decrease in the content of total GSH in the blood of the chickens.

Intensified lipid and protein oxidation processes lead to the generation of radicals, which may result in depletion of endogenous antioxidants and an initial increase, often followed by a decrease, in the activity of antioxidant enzymes (Ognik & Krauze 2016). This possibility is indicated by the increase observed in SOD activity and the reduced level of GSH + GSSG. The total content of GSH in the body consists of its reduced fraction, accounting for 98% of the total concentration, and its oxidized fraction. A reduction in the concentration of GSH should be regarded as disadvantageous; it may be the result of its participation in reactions with oxidants. Reduced GSH is used by glutathione peroxidase in removing hydrogen peroxide generated during lipid oxidation. According to Nathan *et al.* (2002), when the body's redox balance is disturbed, the Cu in plasma or tissue may exhibit oxidant properties.

To sum up, the results of the experiment indicate a multi-faceted effect of nano-Cu, both beneficial and detrimental. Correction of the deficient basal diet of chickens with nano-Cu to a level of Cu exceeding the NRC recommendation (1994) by 54% increased the antioxidant potential of the organism and inhibited lipid peroxidation. Only when the addition of nano-Cu increased the total Cu intake to a level exceeding the recommendation by 96% did symptoms of deterioration in antioxidant status appear (a decrease in the level of GSH+GSSG and an increase in SOD, CAT and Cp activity and in LOOH content). On the other hand, an increase of just 7% over the recommended level of Cu in the diet of chickens was followed by unfavorable results, indicating a deterioration in red blood cell parameters and stimulation of the immune system (an increase in IL-6, IgA, IgM and IgY).

Conclusions

In our experiment, in which the basal supplemented diet was Cu-deficient (−29% vs. NRC recommendations; 5.7 vs. 8 mg/kg), we showed that it is possible to simultaneously increase antioxidant and immune defense of chickens by supplementing their diet with nano-Cu – up to 12 mg per bird during 6 weeks of feeding, that is to a level no more than 7% over the NRC recommendation (1994) for growing broiler chickens.

In view of the above, the levels of added nano-Cu should depend on the content of this element in the basal diet. Moreover, since commercially available metal nanoparticles differ in size, in the methods used to produce them, and in their media, the results of the study should not be generalized. For this reason further research in this interesting area is essential.

ACKNOWLEDGMENT

The experiments conducted within Biostrateg program 'GUTFEED - innovative nutrition in sustainable poultry production' 267659/7/NCBR/2015.

REFERENCES

Abdulazeez H, Adamu SB, Igwebuike JU, Gwayo GJ, Muhammad AI. 2016. Haematology and Serum Biochemistry of Broiler Chickens Fed Graded Levels of Baobab (*Adansonia digitata* L.) Seed Meal. *IOSR Journal of Agriculture and Veterinary Science (IOSR-JAVS)* **9**, 48–53.

Aebi H. 1984. Catalase in vitro. *Methods in Enzymology* **105**, 121–126.

Angelova M, Asenova S, Nedkova V, Kolarova-Koleva R. 2011. Copper in the human organism. *Trakia Journal of Sciences* **9**, 88–98.

Bomski H. 1995. Biernacki's reaction. In: Bomski H (ed.), *Basic Hematology Laboratory Analyses*. National Institute of Medical Publications, Warsaw, Poland. (In Polish)

Brewer GJ. 2007. Iron and copper toxicity in diseases of aging, particularly atherosclerosis and Alzheimer's disease. *Experimental Biology and Medicine* **232**, 323–335.

Cherukuri S, Tripoulas NA, Nurko S, Fox PL. 2004. Anemia and impaired stress-induced erythropoiesis in aceruloplasminemic mice. *Blood Cells, Molecules and Diseases* **33**, 346–355.

Dinant HJ, Dijkmans BAC. 1999. New therapeutic targets for rheumatoid arthritis. *Pharmacy World & Science* **21**, 49–59.

Dusek P, Jankovic J, Le W. 2012. Iron dysregulation in movement disorders. *Neurobiology of Disease* **46**, 1–18.

Gaetke LM, Chow CK. 2003. Copper toxicity, oxidative stress, and antioxidant nutrients. *Toxicology* **189**, 147–163.

Ghasemipoor M, Zolghadri S. 2014. The effect of copper oxide nanoparticles as feed additive on some the blood proteins of broiler chickens. *Molecular Cell Biology Research Communications* **3**, 144.

Kwiecień M, Winiarska-Mieczan A, Zawiślak K, Sroka S. 2014. Effect of copper glycinate chelate on biomechanical, morphometric and chemical properties of chicken femur. *Annals of Animal Science* **14**, 127–139.

Leeson S. 2009. Copper metabolism and dietary needs. *World's Poultry Science Journal* **65**, 353–366.

Letelier ME, Lepe AM, Faúndez M, Salazar J, Marín R, Aracena P, *et al.* 2005. Possible mechanisms underlying copper-induced damage in biological membranes leading to cellular toxicity. *Chemico-Biological Interactions* **151**, 71–82.

Małaczewska J. 2014. Impact of noble metal nanoparticles on immune system of animals. *Medycyna Weterynaryjna* **70**, 204–208. (In Polish)

Maltais D, Desroches D, Aouffen M, Mateescu MA, Wang R, Paquin J. 2013. The blue copper ceruloplasmin induces aggregation of newly differentiated neurons: A potential modulator of nervous system organization. *Journal of Neuroscience* **121**, 73–82.

Martínez A, Reina M. 2017. Copper or free radical scavenger? *Computational and Theoretical Chemistry* **1104**, 1–11.

Meyer LA, Durley AP, ProhaskaJR HarrisZL. 2001. Copper transport and metabolism are normal in aceruloplasminemic mice. *The Journal of Biological Chemistry* **276**, 36857–36861.

Mikulski D, Jankowski J, Zduńczyk Z, Mikulska M. 2009. Copper balance, bone mineralization and the growth performance of turkeys fed diet with two types of Cu supplements. *Journal of Animal and Feed Sciences* **18**, 677–688.

Miroshnikov SA, Yausheva EV, Sizova EA, Miroshnikova EP. 2015. Comparative assessment of effect of copper Nano and microparticles in chicken. *Oriental Journal of Chemistry* **31**, 2327–2336.

Mitruka BM, Rawnsley HM. 1977. *Clinical, Biochemical and Haematological Reference Values in Normal and Experimental Animals*, Vol. **83**, pp. 134–135. Mason Publishing, USA.

Mroczek-Sosnowska N, Batorska M, Łukasiewicz M, Wnuk A, Sawosz E, Jaworski S, *et al.* 2013. Effect of nanoparticles of copper and copper sulfate administered in ovo on hematological and biochemical blood markers of broiler chickens. *Animal Science Journal* **52**, 141–149.

Nathan EH, Satoshi K, Grazia MM, Hoogeboom AJ, Jong GJ, Jonathan DG. 2002. Mechanisms of copper incorporation into human ceruloplasmin. *The Journal of Biological Chemistry* **277**, 46632–46638.

NRC (National Research Council). 1994. *Nutrient Requirements of Poultry, 9th*, revised edn. National Academic Press, Washington, DC, USA.

Ognik K, Krauze M. 2016. The potential for using enzymatic assays to assess the health of turkeys. *World's Poultry Science Journal* **72**, 535–550.

Ognik K, Wertelecki T. 2012. Effect of different vitamin E sources and levels on selected oxidative status indices in blood and tissues as well as on rearing performance of slaughter turkey hens. *The Journal of Applied Poultry Research* **2**, 259–271.

Ognik K, Stępniowska A, Cholewińska E, Kozłowski K. 2016. The effect of administration of copper nanoparticles to chickens in drinking water on estimated intestinal absorption of iron, zinc, and calcium. *Poultry Science* **95**, 2045–2051.

Palumaa P. 2013. Copper chaperones. The concept of conformational control in the metabolism of copper. *FEBS Letters* **587**, 1902–1910.

Pineda L, Sawosz E, Vadalasetty KP, Chwalibog A. 2013. Effect of copper nanoparticles on metabolic rate and development of chicken embryos. *Animal Feed Science and Technology* **186**, 125–129.

Polińska B, Matowicka-Karna J, Kemona H. 2009. The cytokines in inflammatory bowel disease. *Postępy Higieny i Medycyny Doświadczalnej* **63**, 389–394. (In Polish)

Salih AM. 2010. Serum ceruloplasmin, copper and iron levels as a risk factors for coronary heart diseases (CHD). *Baghdad Science Journal* **7**, 372–381.

Samanta B, Ghosh PR, Biswas A, Das SK. 2011. The Effects of Copper Supplementation on the Performance and Hematological Parameters of Broiler Chickens. *Asian-Australasian Journal of Animal Sciences* **24**, 1001–1006.

Sharma D, Kochar B, Bhardwaj A, Riyat M, Sharma P. 2009. Effect of ingestion of copper bhasm on red cell indices, iron parameters and essential elements in chicks. *Indian Journal of Clinical Biochemistry* **24**, 245–249.

Siwicki AK, Anderson DP. 1993. Nonspecific defence mechanisms assay in fish. II. Potential killing activity of neutrophils and macrophages, lysozyme activity in serum and organs, and total immunoglobulin (Ig) level in serum. In: *Fish Disease Diagnosis and Preventions Methods*. Wydawnictwo Instytutu Rybactwa Środlądowego, Olsztyn, Poland.

Song A, Zhu L, Zhao T, Jiao H, Lin H. 2009. Effect of copper on plasma ceruloplasmin and antioxidant ability in broiler chickens challenged by lipopolysaccharide. *Asian-Australasian Journal of Animal Sciences* **22**, 1400–1406.

Sui F, Zhu L, Zhu F, Chen F, Wng Y, Sun J. 2011. Effects of nano-CuO on contents of copper in blood and liver, the CP mRNA expression of liver in chickens. *Chinese Journal of Veterinary Science* **6**, 903–907.

Sunderman FWJR, Nomoto S. 1970. Measurement of human serum ceruloplasmin by its p-phenylenediamine oxidase activity. *Clinical Chemistry* **16**, 903–910.

Tapiero H, Townsend DM, Tew KD. 2003. Trace elements in human physiology and pathology. *Copper. Biomedicine & Pharmacotherapy* **57**, 386–398.

Wang C, Wang MQ, Ye SS, Tao WJ, Du YJ. 2011. Effects of copper-loaded chitosan nanoparticles on growth and immunity in broilers. *Poultry Science* **90**, 2223–2228.

Wu Z, Fernandez-Lima FA, Russell DH. 2010. Amino acid influence on copper binding to peptides: Cysteine versus arginine. *Journal of The American Society for Mass Spectrometry* **21**, 522–533.

Zerounian NR, Linder MC. 2002. Effects of copper and ceruloplasmin on iron transport in the Caco 2 cell intestinal model. *The Journal of Nutritional Biochemistry* **13**, 138–148.

Zhao J, Riediger M. 2014. Detecting the oxidative reactivity of nanoparticles: A new protocol for reducing artifacts. *Journal of Nanoparticle Research* **16**, 2493.