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THE EFFECT OF ADMINISTRATION OF COPPER NANOPARTICLES IN DRINKING WATER ON REDOX REACTIONS IN THE LIVER AND BREAST MUSCLE OF BROILER CHICKENS*

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Abstract

The aim of the study was to determine what dosage of copper nanoparticles added in the form of a hydrocolloid to standard dietary supplementation with copper sulphate will increase the antioxidant potential in the liver and breast muscle of chickens. In total, 126 one-day-old Ross 308 chickens were assigned to 7 experimental groups of 18 birds each (3 replications of 6 individuals each). The basal-diet treatment did not receive nano-Cu. Experimental groups 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). Dietary supplementation of chickens with nano-Cu to exceed the Cu level recommended by the NRC increased the content of Cu (P=0.042) while reducing that of Zn in the liver (P=0.031) and breast muscle (P=0.036). Supplementing the diet of chickens with nano-Cu to a level exceeding the level of copper recommended by the NRC by 7% to 25% increased the antioxidant potential of the liver and the breast meat. The study has shown that the antioxidant status of the liver and breast meat of chickens can be improved by supplementing the standard dietary copper sulphate supplement with the addition of nano-Cu, but to a level not exceeding 25% of the copper content recommended by NRC (1994) for broiler chickens, but the most safe is the nano-Cu level not exceeding 7% of the copper content recommended by NRC (1994).

Key words: nano-copper, breast muscle, liver, minerals, redox status, chicken

The beneficial effect of Cu on the development and functioning of birds is very well documented in the literature (Xiang-Qi et al., 2009; Collins et al., 2010; Hatori

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and Lutsenko, 2016; Ognik et al., 2016; Kozłowski et al., 2018; Ognik et al., 2018). This element conditions the correct course of many important metabolic processes – it participates in binding of iron to haem in haemoglobin and in synthesis of red blood cells, and is responsible for the structure of connective tissue, ensuring proper collagen cross-linking (Collins et al., 2010). In addition, as a co-factor of many enzymes, Cu determines the correct course of certain metabolic processes, including energy production in the respiratory chain, biosynthesis and degradation of neurotransmitters, or redox signalling in angiogenesis (Hatori and Lutsenko, 2016). As a component of the antioxidant enzyme superoxide dismutase, copper has an active role in the body's antioxidant defence against the effects of free radicals, thereby limiting lipid oxidation reactions (Ognik et al., 2018).

Copper deficiency may result in numerous metabolic and functional disorders, including anaemia, neutropaenia, growth and reproduction disorders, bone damage, heart failure (Aoki, 2004; Soetan et al., 2010), nervous system disorders (Jaiser and Winston, 2010), and the intensification of lipid oxidation (Ognik et al., 2018), and therefore poultry diets require supplementation with this element. The form of copper most commonly added to poultry feed is $CuSO_4$. According to the literature, however, copper from this compound is relatively poorly absorbed by the body and substantial quantities are excreted into the environment (Zhao et al., 2010; Karimi et al., 2011). Therefore, the search continues for an alternative source of Cu which would increase the absorption of this element, decrease the level of supplementation, and reduce emissions of Cu to the environment (Mroczek-Sosnowska et al., 2014; Majewski et al., 2017).

According to NRC (1994) recommendations, the addition of Cu to the diet should be 4 mg/kg of feed for growing chickens and 8 mg/kg for broiler chickens. Current EU law states that the level of copper in the diet of all types of poultry must not exceed 25 mg/kg of diet (EFSA, 2016). Previous research, summarized in a review by Leeson (2009), indicates that a 20-fold increase in the dosage of copper in relation to the requirement for this element may have a health-promoting effect by stimulating the immune and antioxidant system, and that only a hundredfold exceedance of the Cu requirement may have toxic effects. Our own research (Ognik et al., 2018) has shown that the addition of a hydrocolloid of copper nanoparticles to a standard diet supplemented with copper sulphate, exceeding the NRC (1994) recommendation by even 100%, did not affect growth results in chickens. The study cited showed that the antioxidant and immune defence of chickens can be simultaneously improved by the addition of nano-Cu, but only up to a level exceeding the NRC recommendation by 7%.

It is unknown, however, whether the addition of nano-Cu to the standard dietary supplementation with copper sulphate in amounts not exceeding 7% of the copper requirement is sufficient or at all necessary to improve the antioxidant status in the liver and breast meat of chickens (Ognik et al., 2018). Increasing the content of low-molecular and enzymatic antioxidants in poultry meat may significantly reduce undesirable oxidation processes, and thus improve the quality of the product and extend its shelf-life, which is important for the consumer.

Therefore, the aim of the study was to determine what dosage of nano-Cu added in the form of a hydrocolloid to standard dietary supplementation with copper

sulphate will increase the antioxidant potential in the liver and breast muscle of chickens.

Material 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 copper nanoparticles were non-ionic, nanocrystalline, chemically pure particles 5 nm in size, 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. (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 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 compound feeds appropriate for the rearing period, in accordance with feeding standards for poultry (NRC, 1994; Table 1). The experiment was carried out on 126 chickens assigned to seven experimental groups of 18 birds each (3 replications of 6 individuals each).

The experimental treatments, i.e. 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. The control 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 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 its high cost. 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 exceeded the recommended intake by 7% ($T1_{1.0}$) to nearly 100% ($T2_{1.5}$) (Ognik et al., 2018).

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 copper content was determined (Table 1). Taking into account NRC (1994) standards, we calculated the percentage of the requirement for copper received by the chickens

(Table 2). After slaughter, the carcasses were scalded, plucked, and eviscerated. The redox status and content of minerals in the liver and breast muscle were determined for 6 samples collected from each group.

Table 1. Composition of 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[1], 26% protein	40.07	43.58	46.87
Monocalcium phosphate	11.03	5.42	2.05
Coarse-grained fodder chalk[2]	–	10.93	8.52
Fine-grained fodder chalk	16.07	–	–
Sodium chloride	3.63	3.23	2.83
DL-Methionine 99%	3.61	2.40	2.00
L-Lysine HCl	4.27	2.97	3.12
L-Threonine 99%	1.31	0.94	0.82
Premix[3, 4]	5.0	5.0	5.0
Calculated composition of 1 kg of mixture			
Crude protein (g/kg)	210.0	198.5	187.5
Crude fibre (g/kg)	27.2	29.8	32.2
Crude fat (g/kg)	65.9	74.5	81.4
Lysine (g/kg)	13.5	11.7	10.9
Methionine (g/kg)	6.7	5.5	5.0
Methionine + cysteine (g/kg)	10.1	8.8	8.3
Tryptophan (g/kg)	2.5	2.3	2.1
Arginine (g/kg)	13.1	12.1	11.1
Total calcium (g/kg)	9.8	7.3	6.0
Phosphorus available (g/kg)	3.9	2.8	2.1
Sodium (g/kg)	1.6	1.5	1.4
Metabolizable energy (MJ/kg)	12.9	13.2	13.4

[1]DDGS – maize distillers dried grains with solubles.

[2]Calcium carbonate.

[3]Vitamin: **wks 1–3:** retinol, 1034 mg/kg; cholecalciferol, 25 mg/kg; α-tocopherol, 15 000 mg/kg; menadione, 4 mg/kg; thiamine, 3 mg/kg; riboflavin, 8 mg/kg; pyridoxine, 5 mg/kg; cobalamin, 16 mg/kg; folic acid, 2 mg/kg; biotin, 0.2 mg/kg; nicotinic amid, 60 mg/kg; calcium pantothenicum, 18 mg/kg; choline, 1.8 g/kg; **wks 4–5:** retinol, 827 mg/kg; cholecalciferol, 25 mg/kg; α-tocopherol, 10 000 mg/kg; menadione, 2 mg/kg; thiamine, 2 mg/kg; riboflavin, 6 mg/kg; pyridoxine, 4 mg/kg; cobalamin, 16 mg/kg; folic acid, 1.75 mg/kg; biotin, 0.05 mg/kg; nicotinic amid, 60 mg/kg; calcium pantothenicum, 18 mg/kg; choline, 1.6 g/kg; **wk 6:** retinol, 827 mg/kg; cholecalciferol, 25 mg/kg; α-tocopherol, 10 000 mg/kg; menadione, 2 mg/kg; thiamine, 2 mg/kg; riboflavin, 5 mg/kg; pyridoxine, 3 mg/kg; cobalamin, 11 mg/kg; folic acid, 1.5 mg/kg; biotin, 0.05 mg/kg; nicotinic amid, 35 mg/kg; calcium pantothenicum, 18 mg/kg; choline, 1.6 g/kg.

[4]Trace minerals: manganese, 100 mg/kg; zinc, 80 mg/kg; iron, 80 mg/kg; copper, 8 mg/kg; iodine, 1 mg/kg; selenium, 0.15 mg/kg; coccidiostat – salinomycin (except wk 6).

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

Item		Treatment						
		BN[4]	$T1_{0.5}$	$T1_{1.0}$	$T1_{1.5}$	$T2_{0.5}$	$T2_{1.0}$	$T2_{1.5}$
Daily dose of nano-Cu hydrocolloids (ml)	wk 2	0	41	41	41	41	41	41
	wk 4	0	128	128	128	128	128	128
	wk 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[1]		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[2] (mg/bird)		24.22	30.28	36.34	42.43	38.36	52.50	66.70
Cu intake in relation to NRC (1994) recommendation (%[3])		–28.7	–11	+7	+25	+13	+54	+96

[1]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 of life.

[2]In group C intake only in feed on days 1–42 of life, in other groups total Cu intake in feed and nano-Cu hydrocolloids.

[3]In accordance with the NRC recommendation (1994) the reference point was a diet containing 8 mg Cu/kg and average consumption of 4.25 kg fodder during days 1–42 of life.

[4]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).

Laboratory analysis

Copper, zinc and iron content in the samples of liver and breast muscle and in the feeds were determined by inductively coupled plasma optical emission spectrometry. As described in a previous work (Ognik and Wertelecki, 2012), the following indicators of antioxidant status were determined in the liver and breast muscle of the chickens: activity of superoxide dismutase (SOD) and catalase (CAT), and the concentration of lipid peroxides (LOOH), malondialdehyde (MDA), reduced glutathione (GSH) and oxidized glutathione (GSSG). Activity of ceruloplasmin (Cp) was determined using a Ceruloplasmin ELISA kit (Biomatik, Delaware, USA).

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 – effect of nano-Cu dose (0.5, 1.0 and 1.5 mg/kg BW per day), T – effect of time (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 by the Newman-Keuls post hoc test at $P \leq 0.05$. The statistical analysis was performed using the GLM procedure for Statistica 8.0PL

software (StatSoft Corp., Kraków, Poland). Treatment effects were considered to be significant at P≤0.05. All data were expressed as means with pooled SE. A Student's t-test was used to compare the slopes of the regression equations.

Results

Our research has shown that the administration of nano-Cu to chickens had no effect on body weight gain (BWG). In BN treatment BWG was 2.33 kg, in $T1_{0.5}$ – 2.34 kg, in $T1_{1.0}$ – 2.35 kg, in $T1_{1.5}$ – 2.37 kg, in $T2_{0.5}$ – 2.34 kg, in $T2_{1.0}$ – 2.33 kg and in $T2_{1.5}$ – 2.32 kg.

Effect of dosage of copper nanoparticles

There was no effect of the nano-Cu dose on the glutathione level in the liver and the breast muscle (Table 6). As the Cu concentration in the hydrocolloid of nano-Cu increased, the content of Cu in the liver of the chickens increased (P=0.042), while the content of Zn in the liver (P=0.031) and breast muscle (P=0.036) decreased (Table 3). Increasing the level of nano-Cu in the diet resulted in a decrease in the level of LOOH in the liver and in the breast muscle (P=0.041 and P=0.048, respectively) and an increase in MDA levels in the liver and breast muscle (P=0.021 and P=0.007, respectively) (Table 4). Increasing the amount of nano-Cu in the diet resulted in a decrease in SOD activity in the breast muscle (P=0.012) and an increase in CAT and Cp activity in the liver and the breast muscle (CAT: P=0.003 and P=0.011, respectively; Cp: P<0.001 and P<0.001, respectively) (Table 5).

Table 3. Copper, zinc, iron content (mg/kg) in the liver and breast muscle of the chickens[1]

Item	Cu		Zn		Fe	
	liver	breast muscle	liver	breast muscle	liver	breast muscle
1	2	3	4	5	6	7
BN	2.12	2.51	7.48	8.762	77.13	6.478
Nano-Cu treated						
$T1_{0.5}$	2.99 b*	2.74 b*	7.49 a	8.443 a	77.58	6.492
$T1_{1.0}$	3.68 a*	2.87 a*	6.84 b*	7.896 ab	78.15	6.547
$T1_{1.5}$	3.99 a*	2.88 a*	6.67 b*	7.679 c*	78.84	6.749
$T2_{0.5}$	3.69 a*	2.89 a*	7.36 ab	8.047 ab	77.45	6.145
$T2_{1.0}$	3.61 a*	2.81 ab*	7.14 ab	7.452 b	78.34	6.349
$T2_{1.5}$	3.96 a*	2.84 a*	6.49 ab*	7.386 b	78.64	6.274
SEM	0.467	0.081	0.236	0.039	0.025	0.018
Dosage effect (D)						
0.5	3.34	2.81	7.42	8.245	77.515	6.318
1.0	3.64	2.84	6.99	7.674	78.245	6.698
1.5	3.97	2.86	6.58	7.532	78.740	6.561

Table 3 – contd.

1	2	3	4	5	6	7
Time effect (T)						
T1	3.55	2.83	7.00	8.006	78.190	6.796
T2	3.75	2.84	6.99	7.628	78.143	6.256
P-value						
D effect	0.042	0.061	0.031	0.036	0.550	0.087
T effect	0.092	0.124	0.154	0.059	0.128	0.136
D × T interaction	0.081	0.067	0.236	0.139	0.349	0.236

[1]see Table 2.

a, b – means within the same column differ significantly (P≤0.05) according to Newman–Keuls mean comparison (only in the case of significant D × T interaction).

* means within the same column differ significantly from the BN at P≤0.05 according to Student's t-test procedure. Data from groups treated with nano-Cu subjected to two-way analysis of variance.

Table 4. Lipid peroxides (LOOH) and malondialdehyde (MDA) content (μmol/g) in the liver and breast muscle of the chickens[1]

Item	LOOH		MDA	
	liver	breast muscle	liver	breast muscle
BN	5.135	20.36	1.315	8.162
Nano-Cu treated				
$T1_{0.5}$	5.089 a	19.65 ab	1.295 c*	8.177 a
$T1_{1.0}$	4.148 b*	19.37 ab	1.269 c*	7.379 b*
$T1_{1.5}$	3.051 c*	17.64 b*	1.236 a*	7.178 a*
$T2_{0.5}$	4.869 ab*	20.02 a	1.103 b*	7.296 ab*
$T2_{1.0}$	4.074 b*	20.28 ab	1.423 b*	8.267 a*
$T2_{1.5}$	4.126 b*	20.07 ab	1.489 b*	8.947*
SEM	0.236	0.079	0.018	0.155
Dosage effect (D)				
0.5	4.979	19.83	1.199	7.736
1.0	4.111	19.82	1.346	7.823
1.5	3.588	18.85	1.362	8.062
Time effect (T)				
T1	4.096	18.88	1.266	7.578
T2	4.356	20.12	1.338	8.170
P-value				
D effect	0.041	0.048	0.021	0.007
T effect	0.038	0.026	0.017	0.004
D × T interaction	0.057	0.876	0.247	0.015

[1]see Table 2.

a, b – means within the same column differ significantly (P≤0.05) according to Newman–Keuls mean comparison (only in the case of significant D × T interaction).

* means within the same column differ significantly from the BN at P≤0.05 according to Student's t-test procedure. Data from groups treated with nano-Cu subjected to two-way analysis of variance.

Table 5. Activity of superoxide dismutase (SOD), catalase (CAT) and ceruloplasmin (Cp) (U/g protein) in the liver and breast muscle of the chickens[1]

Item	SOD		CAT		Cp	
	liver	breast muscle	liver	breast muscle	liver	breast muscle
BN	15.19	59.67	39.62	87.36	0.046	0.021
Nano-Cu treated						
$T1_{0.5}$	14.36 b*	53.32 a	44.39 c*	86.38 ab*	0.097 d*	0.031 e*
$T1_{1.0}$	14.69 b*	55.41 a	48.34 bc*	87.25 ab*	0.112 d*	0.088 d*
$T1_{1.5}$	14.39 b*	49.58 b	47.52 bc*	88.30 a*	0.138 bc*	0.148 a*
$T2_{0.5}$	14.12 b*	58.63 a	42.36 c	82.36 b	0.127 c*	0.069 c*
$T2_{1.0}$	15.38 a*	42.36 b*	51.47 b*	84.13 ab*	0.154 a*	0.133 b*
$T2_{1.5}$	15.89 a*	41.31 b*	62.37 a*	98.60 a*	0.147 b*	0.140 a*
SEM	0.236	0.199	0.308	0.088	0.173	0.333
Dosage effect (D)						
0.5	14.48	55.97	43.37	84.37	0.112	0.063
1.0	15.03	48.88	49.90	85.69	0.133	0.110
1.5	15.14	40.44	54.94	93.45	0.147	0.144
Time effect (T)						
T1	14.48	52.77	46.75	87.31	0.115	0.089
T2	15.13	44.10	52.06	88.36	0.142	0.123
P-value						
D effect	0.086	0.012	0.003	0.011	<0.001	0.001
T effect	0.063	0.008	0.041	0.115	<0.001	0.024
D × T interaction	0.057	0.046	0.057	0.071	0.052	0.147

[1]see Table 2.

a, b – means within the same column differ significantly (P≤0.05) according to Newman–Keuls mean comparison (only in the case of significant D × T interaction).

* means within the same column differ significantly from the BN at P≤0.05 according to Student's t-test procedure. Data from groups treated with nano-Cu subjected to two-way analysis of variance.

Table 6. Level of reduced glutathione (GSH) and oxidized glutathione (GSSG) (μmol/g) in the liver and breast muscle of the chickens[1]

Item	GSH		GSSG	
	liver	breast muscle	liver	breast muscle
1	2	3	4	5
BN	3.125	1.365	0.352	0.141
Nano-Cu treated				
$T1_{0.5}$	3.164 c	1.406 bc	0.334	0.139 ab
$T1_{1.0}$	3.236 b*	1.428 b*	0.326	0.137 b*
$T1_{1.5}$	3.287 a*	1.395 c	0.308	0.134 b*
$T2_{0.5}$	3.245 b*	1.452 a*	0.393	0.141 a
$T2_{1.0}$	3.085 d*	1.419 b*	0.348	0.146 a
$T2_{1.5}$	3.047 d*	1.428 b*	0.322	0.148 a*
SEM	0.145	0.632	0.038	0.022

Table 6 – contd.

1	2	3	4	5
Dosage effect (D)				
0.5	3.204	1.429	0.363	0.140
1.0	3.160	1.423	0.337	0.141
1.5	3.167	1.411	0.315	0.141
Time effect (T)				
T1	3.229	1.409	0.322	0.136
T2	3.125	1.433	0.354	0.145
P-value				
D effect	0.067	0.112	0.072	0.077
T effect	0.082	0.218	0.084	0.057
D × T interaction	0.150	0.104	0.074	0.239

[1]see Table 2.

a, b – means within the same column differ significantly (P≤0.05) according to Newman–Keuls mean comparison (only in the case of significant D × T interaction).

* means within the same column differ significantly from the BN at P≤0.05 according to Student's t-test procedure. Data from groups treated with nano-Cu subjected to two-way analysis of variance.

Effect of time of application of copper nanoparticles

There was no effect of time of application of nano-Cu on the level of glutathione in the liver and the breast muscle (Table 6). The longer period of application of nano-Cu to chickens (which relatively increased Cu intake) caused a decrease in Zn content in the breast muscle (P=0.059) (Table 3). Compared with the T1 treatments, the liver and breast muscle of the chickens from the T2 procedures had higher levels of LOOH (P=0.038 and P=0.026, respectively) and MDA (P=0.017 and P=0.004, respectively) (Table 4). When the period of administration of nano-Cu was extended (T2), there was a decrease in SOD activity in the breast muscle (P=0.008) and an increase in CAT activity in the liver (P=0.041) and Cp activity in the liver and breast muscle (P<0.001 and P=0.024, respectively) (Table 5).

Effect of total intake of copper: $CuSO_4$ and nano-Cu

In the treatments where the addition of nano-Cu reduced the Cu deficiency from 29 to 11% or increased intake of this element by up to 25% relative to the amount recommended in NRC (1994), the increase in Cu content in the liver and breast muscle was expressed by simple regression equations with very high coefficients of determination, R^2=0.960 and R^2=0.866, respectively. In the treatments where the addition of nanoparticles increased Cu intake to above NRC (1994) recommendations, a linear decrease in Zn content was noted in the liver and breast muscle, described by highly significant regression equations (R^2=0.800, R^2=0.824, respectively). In treatments $T1_{0.5}$, $T1_{1.0}$, $T2_{0.5}$ and $T1_{1.5}$, a linear decrease in the LOOH level was observed in the liver and breast muscle (R^2=0.895 and R^2=0.940, respectively) and in MDA (R^2=0.998 and R^2=0.874, respectively), and a linear increase in GSH content in the liver (R^2=0.988). In addition, in the treatments that increased (even by 52.5%) the intake of copper to above NRC (1994) recommendations, there was a linear increase in Cp activity in the liver and breast muscle (R^2=0.887 and R^2=0.923, respectively) (Table 7).

Table 7. Effect of total copper intake on linearity of changes in selected liver and breast muscle parameters

Item		Regression equation	Comment
Cu (mg/kg)	Liver	y=0.1038x–0.2627; R^2=0.960	Linear increase in Cu content in liver in the range of 24.22–42.43 mg Cu intake
Zn (mg/kg)	Breast muscle	y=0.0203x+2,0795; R^2=0.866	Linear increase in Cu content in breast muscle in the range of 24.22–42.43 mg Cu intake
	Liver	y=–0.052x+8.7728; R^2=0.800	Linear decrease in Zn content in liver in the range of 24.22–52.50 mg Cu intake
LOOH (µmol/g)	Breast muscle	y=–0.0325x+9.3033; R^2=0.824	Linear decrease in Zn content in breast muscle in the range of 24.22–66.70 mg Cu intake
	Liver	y=–0.1186x+8.3058; R^2=0.895	Linear decrease in LOOH content in liver in the range of 24.22–42.43 mg Cu intake
MDA (µmol/g)	Breast muscle	y=–0.1885x+25.786; R^2=0.940	Linear decrease in LOOH content in breast muscle in the range of 24.22–42.43 mg Cu intake
	Liver	Y=–0.0046x+1.4362; R^2=0.998	Linear decrease in MDA content in liver in the range of 24.22–38.36 mg Cu intake
Cp (U/g protein)	Breast muscle	Y=–0.064x+9.8381; R^2=0.874	Linear decrease in MDA content in breast muscle in the range of 24.22–42.43 mg Cu intake
	Liver	Y=0.0037x–0.0244; R^2=0.887	Linear increase in Cp activity in liver in the range of 24.22–52.50 mg Cu intake
GSH (µmol/g)	Breast muscle	Y=0.007x–0.1626; R^2=0.923	Linear increase in Cp activity in breast muscle in the range of 24.22–42.43 mg Cu intake
	Liver	Y=0.0091x+2.8988; R^2=0.988	Linear increase in GSH content in liver in the range of 24.22–42.43 mg Cu intake

LOOH – lipid hydroperoxides; MDA – malondialdehyde; SOD – superoxide dismutase; CAT – catalase; Cp – ceruloplasmin; GSH – reduced glutathione; GSSG – oxidized glutathione.

Discussion

Many studies have shown that when the addition of Cu in the diet of chickens is increased, accumulation of this element increases in tissues (Luo et al., 2005; Almansour, 2006; Samanta et al., 2011; Mroczek-Sosnowska et al., 2014; Adegbenjo et al., 2014). These reports are confirmed by the results of our research, which showed a linear increase in the content of Cu in the liver and breast muscle of chickens when intake of this element was increased (up to 25%) in relation to the level recommended in NRC (1994). Our study also showed that a further increase in the addition of Cu, by 54 or 96% in relation to the amount recommended in NRC (1994), did not cause a linear increase in the content of this element in the liver or breast muscle. Cu taken in with the diet is absorbed and then stored in the body in amounts that will ensure normal metabolism. When the level of copper is too high, its absorption and accumulation probably decrease while its excretion increases. Mroczek-Sosnowska et al. (2014), following a single *in ovo* application of a solution of 50 ppm of Cu in the form of $CuSO_4$ or Cu-NP, showed that the largest amounts of copper, irrespective of the form used, accumulate in the soft organs of hatched chicks, especially in the liver, while the least Cu accumulates in the muscles.

There are reports indicating that copper may interact with other elements due to its occurrence at various levels of oxidation. Interactions between copper and other minerals, such as zinc and iron, are often described as classical examples of mineral interactions that affect the availability of these elements (Ognik et al., 2016). The results of our study showed that increasing the addition of copper in the chickens' diet to almost 100 times the amount recommended by the NRC (1994) resulted in an inversely proportional reduction in Zn content in the liver and breast muscle. The results are consistent with literature reports indicating antagonism between copper and zinc in animals (Ao et al., 2009). It is likely that a negative interaction between these elements already takes place at the level of intestinal absorption. These two elements may compete in the enterocytes to bind to metallothioneins responsible for maintaining homeostasis between minerals. Ognik et al. (2016) showed that administration of nano-Cu to chickens inhibited absorption of zinc in the intestines. The content of zinc in the tissues is regulated by low-molecular-weight proteins rich in cysteine residues called metallothioneins. One molecule of this protein can bind to seven Zn^{2+} ions and as many as twelve Cu^{+} ions (Bjorklund, 2013). Excess copper in the diet and increased binding of copper to metallothioneins may also lead to an imbalance in the zinc concentration in cells and intercellular spaces. Disturbances in the proportions of zinc and copper can lead to metabolic disorders (Ognik et al., 2016). During absorption in the small intestine, copper may also interact with iron, interfering with its absorption (Arredondo and Nunez, 2005). In a study carried out by Skrivan et al. (2005), the addition of copper to the diet of laying hens to above NRC (1994) recommendations resulted in increased Fe accumulation in the liver. Ognik et al. (2016) found no negative effect of nano-Cu on iron absorption. Similarly, in the present study, increasing the amount of copper in the diet of chickens had no effect on the content of iron in the liver and the breast muscle. It is likely that nano-Cu are oxidized to Cu^{+} in larger amounts than to Cu^{2+}, and in this form they are absorbed

via Ctr1 channels. Iron in the form of Fe^{2+} is absorbed through DMT1 channels for divalent metals (Barrett et al., 2010). Due to their different oxidation levels, these elements do not then compete for an absorption path.

Ceruloplasmin, as an acute phase protein synthesized in the liver, is able to bind as many as six Cu atoms absorbed into the bloodstream, irrespective of the oxidation state of this element. Ceruloplasmin transports Cu bound in the plasma to the liver and other organs (Hellman and Gitlin, 2002; Linder, 2016). About 60–90% of the Cu present in the body is in the form of a complex with this protein (Song et al., 2009). Pan and Loo (2000) found that a Cu deficit reduces the content of this element in various organs and decreases the activity of copper-dependent enzymes such as ceruloplasmin and Cu-SOD. Song et al. (2009), feeding broiler chickens a diet containing an additive of 8 or 50 mg Cu/kg diet, showed that the larger addition of Cu to the diet more efficiently increased the plasma activity of ceruloplasmin than the smaller amount. In our research, in the treatments in which the addition of nano-Cu reduced the Cu deficiency from 29 to 11% or increased intake of this element by up to 66% in relation to the amount recommended by the NRC (1994), plasma ceruloplasmin activity was shown to increase (Ognik et al., 2018). Similarly, in the present study, as the dosage of nano-Cu in the diet was increased, ceruloplasmin activity in the liver and breast muscle increased as well. Because ceruloplasmin performs antioxidant functions in the body, increasing its activity through administration of nano-Cu to chickens in their diet should be considered a beneficial effect (Song et al., 2009).

Literature data indicate that a Cu deficiency in the body can significantly impair the body's antioxidant defence system (Gaetke and Chow, 2003). This element is part of the active centres of numerous enzymes, including copper-zinc superoxide dismutase (Cu, Zn-SOD), which plays an important role in antioxidant defence. In addition, reduced glutathione (GSH) forms ligands with Cu that are readily absorbed and thus improve antioxidant defence (Gaetke and Chow, 2003; Pastore et al., 2003). However, excess copper in the body may result in an increase in the amount of free, unbound Cu, which may lead to the generation of reactive oxygen species, as Cu may induce Haber-Weiss and/or Fenton reactions (Gaetke and Chow, 2003; Letelier et al., 2010). These reactions may lead to increased lipid peroxidation and the formation of reactive oxidation products that damage cellular structures (Videla et al., 2003; Linder, 2016). In the present study, in the treatments in which the addition of nano-Cu increased intake of this element by about 25% in relation to NRC (1994) recommendations, we observed a decrease in LOOH and MDA content and SOD activity and an increase in the content of GSH. We obtained different results when the addition of nano-Cu increased the intake of this element by about 50% or more in relation to the amount recommended by the NRC (1994), i.e. an increase in MDA content and SOD activity and a reduction in the content of reduced GSH. The study also shows that even the addition of nano-Cu increasing copper in the diet by just 7% in relation to NRC (1994) recommendations resulted in an increase in CAT activity, and a further increase in the addition of Cu further increased the activity of this enzyme. The results of our research suggest that the addition of a hydrocolloid of nano-Cu to standard dietary supplementation with copper sulphate in amounts not exceeding 25% of the amount recommended by the NRC (1994) beneficially stimulates anti-

oxidant defence mechanisms. However, further increasing the addition of nano-Cu to the diet may result in increased oxidative reactions. Bozkaya et al. (2001) found that a Cu deficiency in the diet of chickens increased induction of lipoperoxidation, in which an increase in MDA content, a decrease in copper-dependent SOD activity, and an increase in CAT activity were observed. Xiang-Qi et al. (2009) found that by increasing the addition of copper to the diet of chickens to 350 mg/$CuSO_4$ kg, lipid oxidation processes in the liver of birds can be favourably inhibited. On the other hand, Ajuwon et al. (2011) found that the addition of 250 mg $CuSO_4$/kg can increase lipid oxidation in birds. The authors reported that the content of MDA increased and the activity of SOD, CAT and GSH+GSSG decreased in the liver of birds. Zhang et al. (2000), who gave rats Cu in excess of recommendations, similarly observed an increase in the MDA level in the serum and liver, a reduction in SOD activity in the erythrocytes, and an increase in Cu content in the hepatocytes. The slightly different results of our research in relation to those presented by other authors (Xiang-Qi et al., 2009; Ajuwon et al., 2011) may be due to the use of different forms of Cu. In our research, the amount of Cu in the diet was increased by adding a hydrocolloid of nano-Cu. The research presented by Pineda et al. (2013) showed that the biological effect of nanoparticles can be completely different to that of the commonly used Cu in the form of sulphate. Due to their smaller size, nano-Cu have greater potential for direct contact with the target cells, so they may display different biological reactivity to that of Cu macromolecules.

In summary, the results of the experiment indicate that supplementing the diet of chickens with nano-Cu to exceed the Cu level recommended by the NRC (1994) increased the content of Cu while reducing that of Zn in the liver and breast muscle. Supplementing the diet of chickens with nano-Cu to a level exceeding the level of copper recommended by the NRC by 7 to 25% increased the antioxidant potential of the liver and the breast meat. Only when the addition of nano-Cu exceeded the amount of Cu recommended by the NRC by about 54% did symptoms of Cu-induced oxidation reactions appear.

Conclusions

The study has shown that the antioxidant status of the liver and breast meat of chickens can be improved by supplementing the standard dietary copper sulphate supplement with the addition of copper nanoparticles, but to a level not exceeding 25% of the copper content recommended by NRC (1994) for broiler chickens. However, according to our previous study it is safest to provide the standard dietary copper sulphate supplement with the addition of copper nanoparticles to a level not exceeding 7% of the Cu content recommended by NRC (1994) for broiler chickens.

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