



## EFFECT OF DIFFERENT LEVELS OF COPPER NANOPARTICLES AND COPPER SULFATE ON MORPHOMETRIC INDICES, ANTIOXIDANT STATUS AND MINERAL DIGESTIBILITY IN THE SMALL INTESTINE OF TURKEYS\*

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### Abstract

It was hypothesized that dietary copper (Cu) nanoparticles, as a substitute for the commonly used copper sulfate, could contribute to lowering the dietary inclusion levels of Cu without compromising growth performance or reducing Cu digestibility and utilization in turkeys. An experiment was carried out on 648 one-day-old Hybrid Converter turkeys divided into 6 groups with 6 replicates per group in a two-factorial design with 3 dietary inclusion levels of Cu (20, 10 and 2 mg kg<sup>-1</sup>) and 2 dietary sources of Cu, copper sulfate and Cu nanoparticles (Cu-SUL and Cu-NPs, respectively). The apparent digestibility coefficients of minerals were determined after 6 weeks, and tissue samples were collected after 14 weeks of experimental feeding. A decrease in the dietary inclusion levels of Cu from 20 to 10 and 2 mg kg<sup>-1</sup> did not reduce the body weights of turkeys at 42 and 98 days of age. In comparison with the remaining treatments, the lowest dietary inclusion level of Cu significantly decreased MDA concentrations in small intestinal tissue (P=0.002) and in the bursa of Fabricius (P=0.001). The replacement of Cu-SUL with Cu-NPs differentially modulated the redox status of selected tissues, i.e., enhanced SOD activity in small intestinal tissue (P=0.001) and decreased total glutathione levels in the bursa of Fabricius (P=0.005). In general, neither the different levels nor sources of additional dietary Cu (main factors) exerted negative effects on the histological structure of the duodenum and jejunum in turkeys. The intestinal digestibility of Cu

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increased with decreasing dietary Cu levels, and as a consequence, the highest apparent digestibility coefficient of Cu (and zinc) was noted in turkeys fed diets with the addition of 2 mg kg<sup>-1</sup> Cu-NPs. Therefore, the environmental burden of excreted Cu was substantially reduced along with decreasing dietary Cu levels but it did not depend on the Cu source.

**Key words:** copper, nanoparticles, small intestine, redox status, turkeys

In modern poultry farming, diets are routinely supplemented with copper (Cu) compounds to meet the requirements of intensively reared birds and improve their health. The results of many experiments show that Cu added to poultry diets at increased doses (above 150 mg kg<sup>-1</sup>) stimulates growth and laying performance (Samantha et al., 2011; Lim and Paik, 2006; Jegede et al., 2012). One of the negative consequences of high dietary inclusion levels of Cu, particularly in the form of sulfate, is increased Cu excretion, which leads to environmental mineral pollution (Bao et al., 2007). To address this problem, organic sources of dietary Cu should be used, such as chelates combined with amino acids, which are characterized by higher bioavailability and can help reduce the excretion of environmental contaminants (Karimi et al., 2011). Nanoparticles, including Cu nanoparticles (Cu-NPs), can also be used as feed additives to improve digestion and absorption in livestock (Bunglavan et al., 2014; Gangadoo et al., 2016; Hill and Li, 2017; Sawosz et al., 2018; Scott et al., 2018; Anwar et al., 2019). An experiment performed on piglets revealed that dietary supplementation with 50 Cu nanoparticles improved growth performance, increased availability and reduced faecal Cu excretion compared with those of the Cu-SUL group (Gonzales-Eguia et al., 2009). A recent study on model rats showed that the use of a reduced dose of Cu vs. the recommended dose positively affected the redox status of the body, and the use of copper nanoparticles vs. common CuCO<sub>3</sub> beneficially protected proteins and DNA against oxidation and nitration processes (Jóźwik et al., 2018; Ognik et al., 2019 a, b; Otowski et al., 2019).

Intestinal mucus and the intestinal walls are the first barriers through which ingested NPs, including Cu-NPs, must pass (Crater and Carrier, 2010). It has been reported that mucin interaction with adhesive NPs can disrupt the “bottle-brush” architecture of mucus (McGill and Smyth, 2010), and metal NPs can be trapped in intestinal mucus by adhesive interactions (Jachak et al., 2012). In addition, different doses and/or Cu activity can induce changes in the microbiota population and indirectly affect mucosal histology (Johnson et al., 1985; Awad et al., 2009). Studies on polystyrene nanoparticles show that small particles are capable of being taken up by the villus epithelium and may enter the bloodstream directly or via the lymph system, where they are then predominantly scavenged by the liver and spleen (Jani et al., 1990; Hillery et al., 1994). Majewski et al. (2017) showed a strong relationship between dietary copper nanoparticles and CuCO<sub>3</sub> salt in the tensile strength of the thoracic aorta, and those effects were attributed to the nanoparticles rather than the copper itself.

Due to their small size, ranging between 1 and 100 nm (10<sup>-9</sup>–10<sup>-7</sup> m), nanoparticles are characterized by a relatively large surface area (Chen et al., 2006; Albanese

et al., 2012; Scott et al., 2018). It was found that the relatively large surface area of Cu-NPs (25 nm) contributed to their higher toxicity compared with that of micro-copper particles (17  $\mu\text{m}$ ) and cupric ions ( $\text{CuC}_{12}\cdot 2\text{H}_2\text{O}$ ) (Chen et al., 2006). Toxicological and feeding tests with high Cu-NP doses have revealed the resulting dysfunction of many organs and tissues in mice and rats (Chen et al., 2006; Cholewińska et al., 2018 b). In feeding trials with low levels of Cu-NP, Cu exerted no adverse effect on the experimental animals and even improved the feed efficiency (Gonzales-Eguia et al., 2009; Cholewińska et al., 2018 a). This is an important consideration since, according to the current EU recommendations, the Cu content of poultry diets should not exceed 25 mg  $\text{kg}^{-1}$  feed (EFSA, 2016). Thus, feed additives should contain highly bioavailable Cu, and research should be continued to evaluate the physiological effects of reducing the dietary inclusion levels of Cu in intensive poultry farming (Ognik et al., 2018).

The aim of this study was to verify the following hypothesis: Cu nanoparticles are characterized by higher bioavailability and biological reactivity *in vivo* than Cu sulfate; therefore, dietary inclusion levels of Cu can be lowered without compromising the gastrointestinal function and growth rate of turkeys. As a result, the amount of Cu in animal nutrition and Cu release into the environment can be reduced.

## Material and methods

A total of 648 one-day-old Hybrid Converter female turkeys purchased from the Grelavi Hatchery in Kętrzyn (Poland) were randomly placed in 36 pens, with 18 birds per pen, with a surface area of 3.7  $\text{m}^2$ . The pens were bedded with wood shavings. The stock density was 4.86 birds/ $\text{m}^2$  until week 6 and 3.2 birds/ $\text{m}^2$  from week 6 until the end of the experiment. Turkeys had free access to feed and water, and age-appropriate management conditions were consistent with the breeding company's recommendations (Hybrid Turkeys, 2014).

Turkeys were divided into 6 groups with 6 replicates per group in a two-factorial design with 3 dietary inclusion levels of Cu (20, 10 and 2 mg  $\text{kg}^{-1}$ ) and 2 dietary sources of Cu: copper sulfate and Cu nanoparticles (Cu-SUL and Cu-NPs). Copper nanoparticles (25 nm in size) in the form of powder (99.8% purity), purchased from Sky Spring Nanomaterials Inc. (USA), were added to a vitamin-mineral premix using a starch carrier. The experiment was approved by the local Ethical Committee for Experiments on Animals in Olsztyn (permission no. 30/2015), and all animals were treated according to EU Directive 2010/63/EU.

The composition of the basal diets, which were supplemented with different doses of Cu, is given in Table 1. Diets were prepared at "Agrocentrum" Feed Mill Ltd. in two stages: (1) basal diets were prepared without the vitamin-mineral premix, and (2) diets for the experimental groups were supplemented with vitamin-mineral premixes containing different levels and sources of Cu, thoroughly mixed, pelleted and crumbled. The birds were weighed at the beginning (day 1) and at 42 and 102 days of age. Feed consumption was monitored on a turkey pen basis. The feed conversion

ratio (FCR, kg of feed per kg of body weight gain) was then calculated. At 102 days of age, 6 birds from each dietary treatment were slaughtered in the experimental slaughterhouse after 8 h of feed withdrawal. The procedure was approved by the Local Animal Care and Use Committee. The birds were electrically stunned (400 mA, 350 Hz), hung on a shackle line and exsanguinated by a unilateral neck cut severing the right carotid artery and jugular vein. After slaughter, the carcasses were scalded, plucked, and eviscerated, and intestinal samples were taken. Three sections were collected for a morphometric analysis of the small intestine, and the bursa of Fabricius and 10 cm segments of the small intestine (starting from Meckel's diverticulum) were collected to determine oxidative stress indices.

Table 1. Composition and nutritional value of experimental turkey diets (g kg<sup>-1</sup>, as-fed basis)

	Experimental period, days		
	1-42	43-70	71-102
Diet composition			
wheat	431.1	462.0	616.6
soybean	389.7	304.6	159.5
faba bean	100.0	100.0	100.0
rapeseed	–	50.0	60.0
soybean oil	28.0	38.6	35.4
sodium sulfate	1.5	1.5	1.5
salt	2.0	1.6	1.7
limestone	16.0	15.7	8.5
monocalcium phosphate	17.5	13.2	6.7
methionine	3.7	2.6	2.0
lysine	4.4	4.0	3.7
threonine	1.2	1.2	0.5
vitamin-mineral premix*	5.0	5.0	4.0
Nutritional value (calculated) <sup>#</sup>			
AME (kcal kg <sup>-1</sup> )	2750	2950	3100
protein	265.0	230.0	185.0
arginine	17.6	15.2	11.8
lysine	17.4	15.0	11.7
methionine	7.1	5.7	4.5
methionine and cysteine	11.3	9.5	7.8
threonine	10.5	9.3	6.8
tryptophan	3.2	2.9	2.2
non-phytin phosphorus	5.5	4.5	3.0
sodium	1.5	1.3	1.3

\*Per kg of diet: vit. A – 24999.75 IU, vit. D – 35000 IU, vit. E – 100 IU, tocopherol – 91 mg, vit. K – 4 mg, vit. B<sub>1</sub> – 5 mg, vit. B<sub>2</sub> – 15 mg, vit. B<sub>6</sub> – 6 mg, vit. B<sub>12</sub> – 0.04 mg, niacin – 100 mg, pantothenic acid – 30 mg, folic acid – 4 mg, choline chloride – 700 mg, calcium d-pantothenate – 32.665 mg, biotin – 0.35 mg, total Se – 0.3 mg, total Fe – 60 mg, total Mn – 100 mg, total Zn – 100 mg, I – 1.5 mg, Ca – 1.0435 g.

<sup>#</sup>The contents of nutrients and non-nutrients were calculated according to the Polish Feedstuff Analysis Tables (Smulikowska and Rutkowski, 2005). The analytically verified protein content in the diets of subsequent feeding periods was 258.8, 225.7 and 179.1 g/kg, respectively.

### Laboratory analyses

As described previously (Ognik and Wertenlecker, 2012), the following indicators of redox status were determined in the small intestinal wall and the bursa of Fabricius of the turkeys: activities of superoxide dismutase (SOD) and catalase (CAT) and concentrations of vitamin C, the sum of reduced and oxidized glutathione (GSH + GSSG), hydroperoxides (LOOH) and malondialdehyde (MDA). The activity of CAT was determined according to Aebi (1984), the activity of SOD was determined using Ransod and Ransel diagnostic kits (Randox Laboratories, Crumlin, UK), and the vitamin C concentration was determined according to Omaye et al. (1979). For MDA determination, 1 g tissue samples were weighed accurately, adding 9 times the volume of PBS (phosphate buffer saline) (0.01 M, pH 7 ~ 7.4) at a weight (g):volume (ml) ratio of 1:9. The samples were homogenized in an ice-water bath and then centrifuged at 10,000 g for 10 min. The supernatant was stored on ice until MDA determination. For the remaining determinations (SOD, CAT, GSH), the tissues were homogenized in PBS (0.01 M, pH 7.4) on ice at a PBS volume (ml):tissue weight (g) ratio of 9:1. The tissue homogenate was centrifuged at 1500 g for 15 minutes, and the supernatant was collected.

To study intestinal morphometry, one square centimetre of whole thickness tissue samples from the duodenum, proximal, middle and distal jejunum was taken. The samples were placed in 4% buffered formaldehyde (Sigma-Aldrich Co., St. Louis, Missouri, USA) for 5 days and then stored in ethanol. The samples were embedded in paraffin, and serial histological sections (5 µm thick) were stained with haematoxylin and eosin for histometric analysis under a light microscope. Villus length, crypt depth, and the thickness of the tunica mucosa and tunica muscularis were measured in 5 to 8 slides for each tissue sample with an optical binocular microscope (OLYMPUS BX 61, Warsaw, Poland) coupled to a digital camera and a PC computer equipped with Cell^P (OLYMPUS) software. Thirty measurements were performed for each analysed parameter.

To investigate the intestinal digestibility of Cu and selected mineral elements in the diets prepared for days 1 to 42, titanium dioxide (TiO<sub>2</sub>) was introduced. On day 42 of the experiment, 6 birds per pen were randomly selected, stunned and sacrificed by cervical dislocation. The ileum was dissected from Meckel's diverticulum to the ileo-ceco-colonic junction, and the digesta was collected from the distal 2/3 for apparent ileal digestibility (AID) determination. Samples of ileal digesta from all birds within each pen were pooled (6 pooled samples per treatment) and immediately frozen (-80°C) until further analysis. The pooled digesta was freeze-dried before chemical analyses. Samples, varying in weight from 25 mg to 500 mg, were digested with 4 mL H<sub>2</sub>SO<sub>4</sub> (SUPRAPUR, Merck) and 2 mL H<sub>2</sub>O<sub>2</sub> (SUPRAPUR, Merck) in closed 50 mL quartz vessels using the Multiwave<sup>®</sup> microwave sample preparation system (Anton Paar Graz, Austria). After cooling, 5.0 mL of an internal standard reagent (Yttrium; 10 mg Y/L) was added and brought up to 1000 mL with DI water. To avoid acid interference effects, the acid concentration of all solutions was identical to that in the digested samples.

The Cu content of diet samples and ileal digesta was determined by inductively coupled plasma optical emission spectroscopy (ICP-OES, Varian Inc., Palo Alto, CA, USA). The contents of zinc (Zn), calcium (Ca), phosphorus (P) and iron (Fe) in

the same samples were determined by flame atomic absorption spectroscopy (FAAS, Varian Inc., Palo Alto, CA, USA). All analyses were performed in triplicate. To analyse the intestinal digestibility of Cu and of the selected mineral elements in the diets prepared for days 1 to 42, titanium dioxide (TiO<sub>2</sub>; Sigma Aldrich, St. Louis, MO) in the amount of 0.5% was introduced, and, later, it was analysed in both feed and digesta samples by ICP-OES (Varian Inc., Palo Alto, CA, USA). The following formula was used for AID calculations:

$$\text{AID of nutrient} = \left\{ 1 - \left[ \frac{\text{concentration of marker in feed}}{\text{concentration of marker in ileum}} \times \frac{\text{concentration of nutrient in ileum}}{\text{concentration of nutrient in feed}} \right] \right\} \times 100\%$$

### Statistical analysis

Two-way ANOVA was performed to determine the effects of the Cu inclusion level (20, 10 and 2 mg kg<sup>-1</sup>) and source (Cu-SUL or Cu-NP) and the interaction between both factors (level × source; L×S). The significance of differences between the mean values of the analysed parameters in groups was estimated by Duncan's multiple range test. The data were processed in the Statistica PL 12.0 application.

## Results

### Dietary Cu content and growth performance

In the first stage of the present experiment (1–42 days), the Cu content of the experimental diets was close to the expected values (Table 2). Minor differences in the content of Cu in the subsequent feeding period could have resulted from differences in the accuracy of the analytical technique as well as from changes in the diet composition. The difference between the total Cu content of the diets and of the supplemental Cu doses indicates that major feed ingredients provided over 10 mg kg<sup>-1</sup> Cu in total.

Table 2. Analysed mineral composition of experimental turkey diets

	Experimental period, days		
	1–42	43–70	71–102
Crude ash (g kg <sup>-1</sup> )	65.0	54.0	47.0
Ca (g kg <sup>-1</sup> )	12.8	11.5	7.10
P (g kg <sup>-1</sup> )	8.70	7.90	5.20
Zn (mg kg <sup>-1</sup> )	172	140	148
Fe (mg kg <sup>-1</sup> )	258	229	221
Cu content of experimental diets* (mg kg <sup>-1</sup> )			
Cu-SUL <sub>20</sub>	31.2	29.1	30.7
Cu-NP <sub>20</sub>	28.4	27.2	26.9
Cu-SUL <sub>10</sub>	21.1	17.9	18.8
Cu-NP <sub>10</sub>	20.4	18.3	17.6
Cu-SUL <sub>2</sub>	14.9	12.6	12.9
Cu-NP <sub>2</sub>	13.7	13.4	12.5

\*Diets supplemented per kg with 2, 10 and 20 mg Cu in the form of copper sulfate (Cu-SUL<sub>2</sub>, Cu-SUL<sub>10</sub>, Cu-SUL<sub>20</sub>) or 2, 10 and 20 mg Cu in the form of nanoparticles (Cu-NP<sub>2</sub>, Cu-NP<sub>10</sub>, Cu-NP<sub>20</sub>).

After 6 and 14 weeks of feeding turkeys diets supplemented with various amounts and sources of Cu, none of the experimental factors affected the body weight of the turkeys or the FCR (Table 3).

Table 3. Growth performance of turkeys fed experimental diets

Parameters	Cu-SUL*			Cu-NP*			SEM	P-value		
	20	10	2	20	10	2		Level (L)	Source (S)	L×S interaction
Body weight										
day 42	2.74	2.69	2.75	2.79	2.73	2.76	0.014	0.246	0.228	0.783
day 102	9.55	9.51	9.43	9.55	9.46	9.56	0.043	0.828	0.733	0.741
FCR (kg/kg)										
days 1–42	1.52	1.56	1.55	1.52	1.54	1.55	0.006	0.149	0.477	0.609
days 1–102	2.18	2.24	2.25	2.21	2.19	2.17	0.009	0.529	0.074	0.052

\*Diets supplemented with 2, 10 and 20 mg of Cu in the form of copper sulfate (Cu-SUL) or 2, 10 and 20 mg of Cu in the form of nanoparticles (Cu-NP)

SEM = standard error of the mean (SD for all birds divided by the square root of the number of birds, n = 36); FCR, feed conversion ratio.

### Antioxidant status of the small intestine

A statistical analysis of the test results revealed that the dietary addition of Cu-NPs caused a significant increase in SOD activity ( $P=0.001$ ) in the small intestinal tissue compared with that of the Cu-SUL treatment (Table 4). In the Cu-SUL treatment, a degree of source interaction was noted: CAT activity in the small intestinal tissue was lowest in turkeys fed diets supplemented with 20 mg kg<sup>-1</sup> Cu, and it significantly increased when doses of 10 and 2 mg kg<sup>-1</sup> were applied ( $P=0.001$ ). In the Cu-NP treatment, the lowest CAT activity was observed in the Cu-NP<sub>10</sub> group ( $P<0.05$  vs. Cu-NP<sub>2</sub>). A degree of source interaction was also noted for the total glutathione (GSH+GSSG) concentration in the small intestinal tissue, which was highest in the Cu-NP<sub>10</sub> group ( $P<0.05$  vs. all other groups). Irrespective of the Cu source, the highest and lowest MDA concentrations in the small intestinal tissue were observed when turkey diets were supplemented with Cu at 20 and 2 mg kg<sup>-1</sup>, respectively ( $P<0.05$ ).

In the bursa of Fabricius, a significant degree of source interaction was noted for the SOD and CAT activities ( $P<0.05$ ) (Table 5). In both cases, only the medium dose (10 mg kg<sup>-1</sup>) of Cu-NPs decreased the SOD and CAT activities compared with those of the Cu-SUL<sub>10</sub> group. In the Cu-SUL treatment, the SOD activity in the bursa of Fabricius was significantly reduced in turkeys fed diets supplemented with 2 mg kg<sup>-1</sup> Cu compared with that of groups Cu-SUL<sub>20</sub> and Cu-SUL<sub>10</sub>. With Cu-NP treatment, a decrease in SOD activity was noted when diets were supplemented with 10 and 2 mg kg<sup>-1</sup> Cu vs. that observed in the Cu-NP<sub>20</sub> group. A degree of source interaction was observed for the CAT activity in the bursa of Fabricius, which was significantly lower at a Cu dose of 10 mg kg<sup>-1</sup>, but not at 20 and 2 mg kg<sup>-1</sup>, vs. that observed for the Cu-SUL treatment. Irrespective of the Cu source, the dietary Cu level of 2 mg kg<sup>-1</sup> was associated with the lowest concentrations of GSH+GSSG and vitamin C in the bursa of Fabricius ( $P<0.05$  vs. 20 and 10 mg kg<sup>-1</sup>). The highest dietary addition of Cu resulted in the lowest GSH+GSSG concentration in the bursa of Fabricius ( $P<0.05$

vs. 20 and 10 mg kg<sup>-1</sup>). A Cu dose of 2 mg kg<sup>-1</sup> contributed to a significant decrease in the MDA concentration in the bursa of Fabricius ( $P < 0.05$  vs. 20 and 10 mg kg<sup>-1</sup>). Regardless of the Cu level, the dietary application of Cu-NPs caused a significant decrease in the total glutathione concentration in this sac-like lymphatic organ in comparison with that of the Cu-SUL treatments.

Table 4. Activity of superoxide dismutase (SOD) and catalase (CAT) and the contents of vitamin C (VIT C), total glutathione (GSH+GSSG), and malondialdehyde (MDA) in the small intestinal wall

Parameters	Cu-SUL*			Cu-NP*			SEM	P-value		
	20	10	2	20	10	2		Level (L)	Source (S)	L×S interaction
SOD (U g <sup>-1</sup> protein)	3.37	4.16	3.88	4.10	3.73	3.87	0.082	0.511	0.001	0.273
CAT (U g <sup>-1</sup> protein)	55.2 c	68.4 a	64.7 ab	65.6 ab	58.1 bc	71.5 a	1.319	0.020	0.299	0.001
GSH+GSSG (μmol kg <sup>-1</sup> )	2.53 b	2.05 b	1.99 b	2.24 b	3.21 a	2.03 b	0.107	–	–	0.007
VIT C (μmol kg <sup>-1</sup> )	154	160	165	150	158	179	3.583	0.074	0.701	0.543
MDA (μmol kg <sup>-1</sup> )	5.78	5.78	4.88	5.93	4.68	4.45	0.148	0.002	0.077	0.145

\*Diets supplemented with 2, 10 and 20 mg of Cu in the form of copper sulfate (Cu-SUL) or 2, 10 and 20 mg of Cu in the form of nanoparticles (Cu-NP).

SEM = standard error of the mean (SD for all birds divided by the square root of the number of birds, n=36); SOD, superoxide dismutase; CAT, catalase; GSH+GSSG, total glutathione; VIT C, vitamin C; MDA, malondialdehyde.

a, b, c – two-way ANOVA applied; among groups, means within the same line with no common letter differed significantly ( $P \leq 0.05$ ) in Duncan's comparison test (calculated only if the L×S interaction was significant).

Table 5. Redox status parameters in the bursa of Fabricius of turkeys

Parameters	Cu-SUL*			Cu-NP*			SEM	P-value		
	20	10	2	20	10	2		Level (L)	Source (S)	L×S interaction
SOD (U g <sup>-1</sup> protein)	9.67 a	9.85 a	8.02 bc	8.94 ab	7.26 c	7.17 c	0.210	–	–	0.023
CAT (U g <sup>-1</sup> protein)	86.6 ab	89.7 a	77.4 b	84.3 ab	65.5 c	77.6 b	1.683	–	–	0.001
GSH+GSSG (μmol kg <sup>-1</sup> )	6.78	8.01	11.2	5.19	7.41	9.67	0.345	<0.001	0.005	0.548
VIT C (μmol kg <sup>-1</sup> )	244	242	254	240	252	255	2.026	0.048	0.523	0.386
LOOH (μmol kg <sup>-1</sup> )	2.72	3.30	2.82	2.71	2.90	3.08	0.106	0.418	0.746	0.466
MDA (μmol kg <sup>-1</sup> )	2.72	2.86	1.85	2.61	2.67	1.68	0.128	0.001	0.511	0.988

\*Diets supplemented with 2, 10 and 20 mg of Cu in the form of copper sulfate (Cu-SUL) or 2, 10 and 20 mg of Cu in the form of nanoparticles (Cu-NP).

SEM = standard error of the mean (SD for all birds divided by the square root of the number of birds, n=36); SOD, superoxide dismutase; CAT, catalase; GSH+GSSG, total glutathione; LOOH, hydroperoxides; MDA, malondialdehyde.

a, b, c – two-way ANOVA applied; among groups, means within the same line with no common letter differed significantly ( $P \leq 0.05$ ) in Duncan's comparison test (calculated only if the L×S interaction was significant).

### Morphometric parameters

In the duodenum, the shortest villi and the lowest mucosa thickness were associated with the diet containing 2 mg kg<sup>-1</sup> supplemental Cu (P<0.05 vs. the 10 mg kg<sup>-1</sup> treatment) (Table 6). An interaction (P=0.048) between the dietary level and source of Cu was found for the thickness of the tunica muscularis in the duodenum: higher thicknesses were noted in the Cu NP<sub>10</sub> and Cu-SUL<sub>2</sub> treatments compared with those of the remaining treatments. In the proximal jejunum, the following degree of source interaction was observed: the thickness of tunica muscularis was highest when turkeys were fed a diet with 10 mg kg<sup>-1</sup> Cu-NPs (P<0.05 vs. all other groups; Table 7). In the distal jejunum, the following degree of source interaction was noted: the application of Cu-NPs at the lowest level significantly decreased crypt depth when compared with that of the Cu-NP<sub>10</sub> group; such a difference was not observed upon the addition of Cu-SUL. Irrespective of Cu level, Cu-NPs increased the thickness of tunica muscularis in the distal jejunum compared with that observed with Cu-SUL treatment.

Table 6. Depth of the crypts of Lieberkühn, the villus height, thickness of the mucosa (mucosa) and thickness of the tunica muscularis (muscle) in the duodenum of turkeys (µm)

Parameters	Cu-SUL*			Cu-NP*			SEM	P-value		
	20	10	2	20	10	2		Level (L)	Source (S)	L×S interaction
Villus height	2478	2679	2395	2421	2477	2370	31.793	0.033	0.120	0.442
Crypt depth	119	132	135	116	127	115	2.782	0.208	0.097	0.375
Mucosa	2610	2825	2545	2517	2619	2501	35.21	0.045	0.093	0.599
Muscle	369 c	410 bc	466 a	364 c	436 ab	406 bc	8.367	–	–	0.048

\*Diets supplemented with 2, 10 and 20 mg of Cu in the form of copper sulfate (Cu-SUL) or 2, 10 and 20 mg of Cu in the form of nanoparticles (Cu-NP).

SEM = standard error of the mean (SD for all birds divided by the square root of the number of birds, n=36).

a, b, c – two-way ANOVA applied; among groups, means within the same line with no common letter differed significantly (P≤0.05) in Duncan's comparison test (calculated only if the L×S interaction was significant).

Table 7. Depth of the crypts of Lieberkühn, the height of villi, thickness of the mucosa (mucosa) and thickness of the tunica muscularis (muscle) in the jejunum of turkeys fed experimental diets (µm)

Parameters	Cu-SUL*			Cu-NP*			SEM	P-value		
	20	10	2	20	10	2		Level (L)	Source (S)	L×S interaction
1	2	3	4	5	6	7	8	9	10	11
Proximal jejunum										
villus height	2612	2382	2285	2502	2350	2441	40.77	0.087	0.955	0.388
crypt depth	125	136	123	124	121	124	2.01	0.625	0.232	0.235
mucosa	2748	2520	2425	2635	2603	2574	37.12	0.100	0.584	0.317
muscle	481 b	438 b	466 b	439 b	559 a	411 b	11.63	–	–	0.001

Table 7 – contd.

1	2	3	4	5	6	7	8	9	10	11
Middle jejunum										
villus height	1550	1649	1597	1557	1668	1658	26.32	0.269	0.595	0.908
crypt depth	146	126	142	141	132	132	3.29	0.230	0.671	0.645
mucosa	1706	1794	1749	1702	1804	1805	26.92	0.350	0.712	0.902
muscle	396	368	434	406	390	413	7.50	0.055	0.817	0.468
Distal jejunum										
villus height	1501	1567	1494	1436	1478	1523	25.53	0.687	0.435	0.632
crypt depth	125 ab	134 a	114 b	114 b	126 ab	126 ab	2.014	–	–	0.029
mucosa	1649	1704	1612	1553	1596	1674	26.30	0.725	0.383	0.357
muscle	437	410	456	513	468	445	8.87	0.189	0.014	0.074

\*Diets supplemented with 2, 10 and 20 mg of Cu in the form of copper sulfate (Cu-SUL) or 2, 10 and 20 mg of Cu in the form of nanoparticles (Cu-NP).

SEM = standard error of the mean (SD for all birds divided by the square root of the number of birds, n=36).

a, b – two-way ANOVA applied; among groups, means within the same line with no common letter differed significantly ( $P \leq 0.05$ ) in Duncan's comparison test (calculated only if the L×S interaction was significant).

### Mineral digestibility

Regardless of the Cu source, the lowest inclusion level of supplemental Cu resulted in a significant increase in the apparent digestibility coefficient of Fe ( $P < 0.05$  vs. 10 and 20 mg  $\text{kg}^{-1}$ ; Table 8). Dietary supplementation with 10 mg  $\text{kg}^{-1}$  Cu led to a significant decrease in the apparent digestibility coefficient of P compared with that of the 20 mg  $\text{kg}^{-1}$  treatment. Irrespective of the Cu level, the dietary application of Cu-NPs caused a significant decrease in the apparent digestibility coefficient of Fe ( $P < 0.05$  vs. the Cu-SUL treatment). Significant levels of source interactions were noted for the apparent digestibility coefficients of Cu and Zn. When Cu-NPs were applied instead of Cu-SUL at 10 and 2 mg  $\text{kg}^{-1}$  (but not at 20 mg  $\text{kg}^{-1}$ ), the apparent digestibility coefficient of Cu was significantly higher. The highest apparent digestibility coefficient of Cu was noted in the Cu-NP<sub>2</sub> group ( $P < 0.05$  vs. all other groups). Similarly, the Cu-NP<sub>2</sub> group was characterized by the highest apparent digestibility coefficient of Zn ( $P < 0.05$  vs. all other groups).

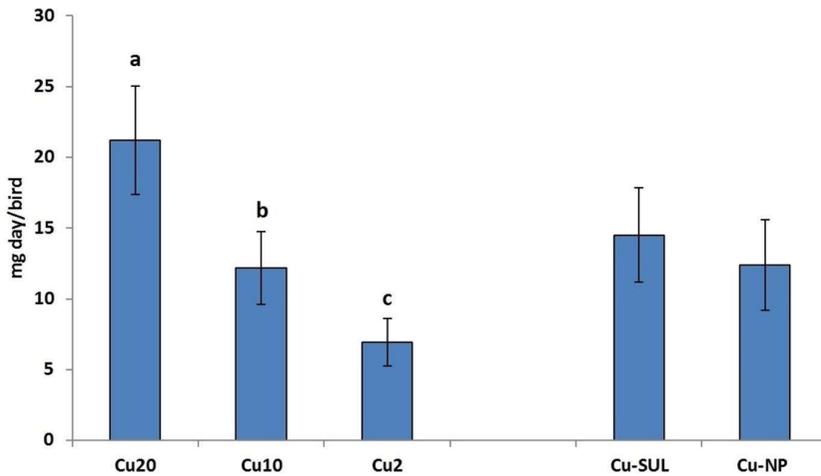
Table 8. Apparent digestibility coefficients of minerals (%)

Parameters	Cu-SUL*			Cu-NP*			SEM	P-value		
	20	10	2	20	10	2		Level (L)	Source (S)	L×S interaction
Cu	23.2 d	27.5 c	36.2 b	26.2 cd	38.3 b	56.3 a	1.704	–	–	<0.001
Zn	25.4 b	29.8 b	24.5 b	28.4 b	25.7 b	47.6 a	1.383	–	–	<0.001
Fe	16.3	18.5	23.5	11.6	13.7	17.7	0.831	<0.001	<0.001	0.926
P	67.4	59.3	66.6	68.4	63.3	61.4	0.924	0.009	0.960	0.083
Ca	56.5	51.3	55.8	50.9	52.1	53.8	0.786	0.252	0.149	0.232

\*Diets supplemented with 2, 10 and 20 mg of Cu in the form of copper sulfate (Cu-SUL) or 2, 10 and 20 mg of Cu in the form of nanoparticles (Cu-NP).

SEM = standard error of the mean (SD for all birds divided by the square root of the number of birds, n=36).

a, b, c, d – two-way ANOVA applied; among groups, means within the same line with no common letter differed significantly ( $P \leq 0.05$ ) in Duncan's comparison test (calculated only if the L×S interaction was significant).



a, b, c – bars with different letters differ significantly ( $P \leq 0.05$ ) in Duncan's comparison test.

Figure 1. Calculated amount of undigested copper excretion (mg/day/bird) in turkeys fed diets with different inclusion levels (mg kg<sup>-1</sup> feed; treatments denoted as Cu<sub>20</sub>, Cu<sub>10</sub>, Cu<sub>2</sub>) of Cu-SUL (copper sulfate) and Cu-NP (copper nanoparticles)

Regardless of the Cu source, the amount of undigested Cu excreted into the environment decreased from 21.2 mg day<sup>-1</sup> bird<sup>-1</sup> in the Cu<sub>20</sub> group to 12.2 and 6.95 mg day<sup>-1</sup> bird<sup>-1</sup> in the Cu<sub>10</sub> and Cu<sub>2</sub> groups, respectively (Figure 1). The replacement of Cu-SUL with Cu-NPs only slightly decreased the amount of undigested Cu (from 14.5 to 12.4 mg day<sup>-1</sup> bird<sup>-1</sup>).

## Discussion

### Dietary Cu content and growth performance

In the first stage of the present experiment (days 1–42), the total Cu content in the three dietary treatments (2, 10, and 20 mg kg<sup>-1</sup>) was lower, similar to and higher, respectively, than the recommended level for poultry diets in the EU, i.e., 25 mg kg<sup>-1</sup> (EFSA, 2016). In the subsequent stages of the study, the total Cu content of the turkey diets was 1 to 2 percentage units lower than the EU-recommended level, most likely due to the lower Cu content of the feed ingredients and an increase in the ground wheat content at the expense of soybean meal. This was consistent with the results of other studies in which basal diets with known Cu contents were supplemented with different amounts of Cu (Mabe et al., 2003; Arias and Koutsos, 2006). According to the literature, supplemental Cu may promote growth in chickens at very high doses, i.e., those reaching 100–450 mg kg<sup>-1</sup> (Pekel and Alp, 2011; Samanta et al., 2011). Makarski et al. (2014) observed no improvement in the growth rate of turkeys at lower dietary Cu levels (15 and 65 mg Cu kg<sup>-1</sup>). Similar results were noted

in our study, where dietary supplementation with Cu-SUL or Cu-NPs had no influence on the growth performance of female turkeys.

### **Antioxidant status of the small intestine**

Research has shown that cellular susceptibility to oxidative damage can be due to both the excess (Ajuwon et al., 2011) and deficiency of dietary Cu (O'Connor, 2001). Dietary Cu is an important part of the body's antioxidant system, and a Cu deficiency reduces the activity of Cu-containing enzymes, including Cu, Zn-SOD and ceruloplasmin (Sukalski et al., 1997). Recent data, presented as another part of the present study, showed that the 20 mg kg<sup>-1</sup> Cu-NP treatment, but not the lower doses, negatively affected the redox status of blood and stimulated the synthesis of the proinflammatory cytokine IL-6 (Jankowski et al., 2019). In the present experiment, the dietary Cu inclusion levels of 20 and 10 mg kg<sup>-1</sup> had no influence on the analysed parameters of the redox status of the small intestinal wall. Interestingly, diets with the lowest level of Cu supplementation (2 mg kg<sup>-1</sup>) caused a decrease in the concentrations of oxidation products, as measured by the MDA level. It can be assumed that the antioxidant balance status of blood, compared with that of internal tissues, seems to be more prone to undesirable changes upon unbalanced (excess or deficiency) dietary Cu levels. Regarding the Cu source, the replacement of Cu-SUL with Cu-NPs increased the SOD activity and tended to decrease MDA levels in the small intestinal wall. The medium dose of supplemental Cu-NPs increased the concentrations of GSH+GSSG but decreased the CAT activity in the small intestinal wall. The above results indicate slight modulatory effects exerted by Cu-NPs on the intestinal redox status, which should be thoroughly examined.

It has been reported that high amounts of supplemental Cu (above 300 mg kg<sup>-1</sup>) decrease the weight and growth index of the bursa of Fabricius, the primary lymphoid organ in avian species (Yang et al., 2009). The effect of lower dietary Cu levels on the size and function of the bursa of Fabricius has not been investigated to date. In the current study, the lowest Cu dietary treatments were found to reduce MDA levels in the bursa of Fabricius. The desirable reduction in MDA concentration may be attributed to higher levels of glutathione and vitamin C in the bursa of Fabricius.

### **Morphometric parameters**

Research has shown that dietary Cu supplementation may affect mucosal histology via alterations of the gut microbiota (Johnson et al., 1985; Awad et al., 2009). In a broiler chicken trial, a dose above 250 mg Cu per kg of feed significantly reduced the villus height and significantly thickened the muscular layer in the duodenum (Chiou et al., 1999). The increased surface area of nanoparticles supports their adhesion to the substrate, deeper penetration into tissues through fine capillaries and prolonged residence of the compounds in the intestines (Chen et al., 2006). It appears that higher dietary levels of Cu-NPs could induce changes in the intestinal epithelium, and there are no published studies involving lower dietary inclusion levels. In the present experiment, certain intestinal morphometric changes were noted, but they did not follow a clear trend depending on the dietary level and source of Cu.

### **Mineral digestibility**

It has been found that changes in essential trace mineral digestibility in the gastrointestinal tract are primary mechanisms for maintaining trace mineral homeostasis (King et al., 2000). In the current study, a decrease in the dietary inclusion levels of Cu from 20 to 10 and then to 2 mg kg<sup>-1</sup> led to a natural increase in the apparent Cu digestibility coefficient. Only in the case of Zn was the highest coefficient of intestinal digestibility observed in the Cu-NP<sub>2</sub> treatment, where Cu digestibility was highest. This indicates that the smallest dose of Cu, including the more readily absorbed form, Cu-NPs, decreased Cu and Zn antagonism in gastrointestinal absorption, as noted in other experiments (Adegbenjo et al., 2014; Ognik et al., 2016). In the case of Fe, digestibility coefficients were higher at the lowest dietary addition of Cu, which corroborates the findings of other authors (Linder and Hazegh-Azam, 1996; Schoendorfer and Davies, 2012), indicating that Cu and Fe compete for transport and bioavailability.

The replacement of Cu-SUL with Cu-NPs increased the apparent digestibility coefficient of Cu in turkeys fed diets with the medium and lowest Cu doses. This finding confirms the opinion that, due to their small size, nanoparticles penetrate more easily through fine capillaries in the intestines (Chen et al., 2006). In our experiment, Cu-NPs increased Zn digestibility (but only when the lowest dietary Cu levels were applied) and decreased Fe digestibility. Research has shown that Cu competes with Fe for intestinal absorption (Linder and Hazegh-Azam, 1996). However, a different relationship has also been reported: Cu deficiency reduces Fe absorption, which is linked to intestinal Fe transport being Cu-dependent (Schoendorfer and Davies, 2012). In view of the above findings, increased Fe digestibility in turkeys fed diets with the lowest Cu dose is indicative of the absence of Cu deficiency symptoms.

In an experiment on piglets, Cu availability was significantly improved, and faecal Cu excretion was reduced in the Cu-NP group compared with that of the Cu-SUL group (Gonzales-Eguia et al., 2009). Other authors have also demonstrated that the use of nanoparticles, including Cu nanoparticles, as feed additives can improve the digestion and absorption of nutrients in livestock (Bunglavan et al., 2014; Gangadoo et al., 2016; Hill and Li, 2017). In our experiment, the replacement of Cu-SUL with Cu-NP decreased the amount of undigested Cu excreted with faeces (from 14.5 to 12.4 mg kg<sup>-1</sup>), thus reducing the environmental burden. However, better results were achieved when the amount of supplemental Cu was decreased from 20 to 10 and 2 mg kg<sup>-1</sup>.

### **Conclusions**

The results of this study indicate that a decrease in the dietary inclusion levels of Cu from 20 mg kg<sup>-1</sup> to 10 and 2 mg kg<sup>-1</sup> did not reduce the body weights of turkeys and had no adverse effect on the morphometric parameters of the small intestine. Surprisingly, the lowest amount of additional Cu (2 mg kg<sup>-1</sup>) improved the antioxidant status of the small intestinal tissue and the bursa of Fabricius. In comparison with Cu-SUL, Cu-NPs did not compromise the growth performance of turkeys and improved selected indicators of the redox status in the host tissues (including an increase in SOD activity in the small intestine and glutathione levels in the bursa

of Fabricius). The lowest amount of additional Cu in the form of nanoparticles increased the apparent digestibility of Cu and Zn. Therefore, the environmental burden of excreted Cu was substantially reduced along with decreasing dietary Cu levels but to a lesser extent when copper sulfate was replaced with Cu nanoparticles. In view of the results of the present study, some of which were quite surprising, further debate is needed to establish the dietary requirements for Cu in turkeys and the efficacy of Cu nanoparticles in poultry nutrition.

### Declarations of interest

None.

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