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ALTERATIONS IN INTESTINAL AND LIVER HISTOMORPHOLOGY AND BASAL HEMATOLOGICAL AND BIOCHEMICAL PARAMETERS IN RELATION TO DIFFERENT SOURCES OF DIETARY COPPER IN ADULT RATS*

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Abstract

Copper (Cu) is required for all basic biochemical and physiological processes. The aim of this study was to evaluate the effects of different sources of dietary Cu on the histomorphometry of liver and jejunal epithelium in adult rats. Male 12-week-old rats were used in a 12-week experiment. The control diet provided the required Cu level from sulfate, and other two diets were supplemented with Cu as a glycine complex at 75% and 100% of daily requirement. Basal hematological and plasma biochemical analyses were also performed. There was no effect of Cu supplementation on the liver weight and the plasma and liver Cu concentration. Histomorphometric analysis of liver tissue showed an increase in the collagen amount and intracellular space in the group supplemented with Cu amino acid. Cu given in the organic form at 100% of daily requirement decreased the muscular and submucosa layer and the crypt depth. In turn, organic copper given at 75% of daily requirement did not influence the intestinal morphology. Dietary Cu given to adult rats as copper sulfate or a glycine complex meeting 100% of the daily requirement appears to be less harmful with regard to intestinal epithelium than when given as a glycine complex at 100% of daily requirement.

Key words: copper, Cu-Gly, liver histomorphometry, jejunum, biochemical parameter, adult rat

Copper (Cu) is the third most abundant essential trace element in the body, after iron and zinc, needed for maintenance of the health of domestic animals (Peňa et al., 1999; Rinaldi, 2000; Linder and Hazegh-Azam, 1996; Fields et al., 1984; Linder and Hazegh-Azam, 1996; Brewer, 2010; Ding et al., 2011; Arakeri and Brennan,

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2013). On the other hand, Cu inhibits the transport and bioavailability of iron, and uptake thereof is competitively inhibited by manganese, zinc, and cobalt (Linder and Hazegh-Azam, 1996). Furthermore, as an ion, Cu is toxic (Brewer, 2010). The liver Cu concentration can be elevated to concentrations that cause toxicosis, damage, and degeneration of liver tissue like in Wilson's disease (Linder and Hazegh-Azam, 1996; Roberts and Michael, 2008).

However, common feed ingredients are usually deficient in Cu, thus commercial diet or additives should provide the essential amount of Cu in a biologically active form, which depends on the physical and chemical properties of the form of the additive in which the trace element is given in the diet (Świątkiewicz et al., 2001; Männer et al., 2006). In supplementation, Cu sources used for animals and humans are divided into inorganic sources, such as copper sulfate or carbonate, and organic sources, such as those offered as a chelate, a specific complex of amino acid with ions. Inorganic salt has additionally poor bioavailability through the presence of ingredients that could impair absorption (the occurrence of phytate, oxalate, or fiber). Thus, inorganic trace mineral administration to farm animals poses a risk for the environment by excretion of high mineral levels, which is not allowed by European Union regulations. An amino acid complex ensures higher bioavailability of Cu, the absorption of which from the small intestine is enhanced by amino acids (Männer et al., 2006). Normally, approximately 50% of ingested Cu is absorbed (Linder and Hazegh-Azam, 1996). Studies in humans and animals indicate that the absorption is regulated by the nutritional status and it depends on the chemical form in which the microelement is present (Świątkiewicz et al., 2001). Moreover, an appropriate dose of Cu-Gly, Fe-Gly or Zn-Gly in the diet of broiler chickens does not reduce their production results, hematological parameters, or the content of trace minerals in the liver (Kwiecień et al., 2015 a, b; Kwiecień et al., 2016 a, b). The number of studies of the chelated form in relation to nutrition of domestic animals seems to be unsatisfactory.

Even though the role of Cu in animal and human health is well established, there is no knowledge about the influence of different sources thereof in the diet on the intestinal epithelium with a mature barrier in adult rats. Thus, we attempted to check whether the administration of Cu as an amino acid chelate would not adversely affect the histomorphometric image of the intestinal epithelium and liver in relation to the Cu administration in the form of $CuSO_4$.

Material and methods

The experimental procedures used throughout this study were approved by the Local Ethics Committee on Animal Experimentation of the University of Life Sciences of Lublin, Poland. The rats were maintained in an animal house according to the guidelines of this committee. Experiment complied with the Guiding Principles for Research Involving Animals.

Animals, breeding and experimental design

Male Wistar rats (n=36) at the age of 12 weeks at the start of the experiment, were used in the experiment that lasted 12 weeks (excluding an acclimatization in the first week). Clinically healthy rats were individually kept in Macrolon cages at 21±1°C and 55% humidity, and 12-hour light and dark cycles. Rats were randomly divided into the control and two experimental groups (each n=12) depending on different levels of organic Cu supplementation. All animals had free access to distilled water (no Cu) and were fed *ad libitum*. The composition of basal diet was: crude protein min. 14.5%, crude fat min. 1.5%, crude fiber min. 5%, ash 10%. The content of vitamin and mineral premixes of the diet is presented in Table 1. The control group was fed with standard diet (LSM, Agropol S.J., Motycz, Poland), which provided the required Cu level for rats in inorganic form (the IN group; 5 mg/kg body weight per day from sulfate ($CuSO_4$)) (Megahed et al., 2013; NCR, 2005). Whereas, other animals fed with the same standard diet, which provided Cu for rats in organic form as Cu amino acid chelate (Cu-Gly, 16% Cu and 37% glycine), were divided into two groups: (1) the OG100 group was fed with the diet, which provided the required Cu level for rats (5 mg/kg body weight per day) and (2) the OG75 group fed with the diet, which covered 75% of daily requirement (3.75 mg/kg body weight per day). The chelated copper covering 100% of daily requirement and the inorganic salts contained an equivalent mineral concentration.

Table 1. Composition of vitamin and mineral premixes of the diet (per kilogram dry matter) fed to rats during the study

Components	Per kg of premix
Manganese (mg)	5 000
Iron (mg)	5 000
Zinc (mg)	2 500
Iodine (mg)	75
Pantothenic acid (D-calcium pantothenate) (mg)	900
Retinol acetate (UI)	800 000
Cholecalciferol (UI)	100 000
Tocopherol (mg)	4 964
Menadione sodium bisulphite (mg)	300
Riboflavin (mg)	600
Pyridoxine HCL (mg)	60
Cyanocobalamin (mg)	1.2

Water consumption was measured weekly, but food consumption daily. At the end of the experiment, rats were fasted for 24 hours and euthanized one by one with carbon dioxide inhalation and by dislocation of the spine.

Hematological and plasma biochemical analyses

Blood samples were collected carefully for hematological and blood plasma biochemical analyses using standard venipuncture of the heart. Hematological analyses were performed with the use of an automatic hematological analyzer MS9 (Melet

Schloesing Laboratories, France). The numbers of white and red blood cells (WBC and RBC), hemoglobin concentration (Hb), and hematocrit (HT) were determined.

The plasma was immediately separated by centrifugation and stored at –25°C for further analysis. The plasma concentration of Cu, Fe and Zn was determined by the colorimetric method using a Metrolab 2300 GL unit (Metrolab SA, Buenos Aires, Argentina) and ready-made sets produced by the company BioMaxima (Lublin, Poland).

Total protein, glucose, total cholesterol, triacylglycerol, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) were determined by the colorimetric method using a Metrolab 2300GL random-access biochemical analyzer (Metrolab SA, Buenos Aires, Argentina) and tests by BioMaxima (Lublin, Poland). Given both activities of AST and ALT, the relationship between AST and ALT was calculated as the de Ritis coefficient. The AST/ALT ratio is commonly useful in differential diagnosis and classification of liver disorders (Cohen and Kaplan, 1975).

Tissue collection and histomorphometrical analysis

A 10 mm long segments from 50% of the total jejunum length were taken from each animal. They were opened along the mesenteric border and pinned flat, without stretching, on pieces of cork in physiological saline. Moreover, the liver samples (0.5 cm^3) were collected at the same place/lobe from each animal. Tissues were fixed in 4% buffered formaldehyde (pH 7.0) for 24 h, dehydrated in graded series of ethanol and embedded in paraffin. The samples obtained from individual animals of each group were subjected to histology, the sections were cut 4 μm thick and were stained using Masson's trichrome method (MT) to differentiate the small intestine wall layers. Microscopic images were collected using a microscope (Axiovert 200M, Carl Zeiss, Jena, Germany). Objective magnifications of 4x, 10x, 20x, and 40x were used to show the different intestinal structures and to collect images of the examined tissues from each specimen for further analysis. The structure of the small intestine wall and liver tissue was examined under microscopic observation and with the use of graphic analysis software Olympus cellSens Version 1.5 (Olympus, Tokyo, Japan).

The following morphometric variables in the intestine were analyzed: mucosa, submucosa and myenteron (longitudinal and transversal lamina) thickness; villar epithelium thickness; enterocyte number per 100 μm of villi; crypt depth (defined as the depth of the invagination between adjacent villi from the bottom of the crypt to the base of villi); crypt width (measured in the middle of the crypt depth); the number of crypts (active: showing mitoses and Paneth cells, having an open internal space and access to the intestinal lumen; inactive: showing no mitoses and Paneth cells, having a closed internal space; total: active plus inactive crypts); villar length (from the tip of the villi to the villous-crypt junction); villar thickness (measured in the middle of villar height); the number of villi; small intestine absorptive surface (Kisielinski et al., 2002).

Microscopic observations allowed identifying and assessing normal structure such as portal triads and terminal hepatic venules, necessary for the evaluation of

the lobular architecture, mature fibrous tissue and portal tract stroma (dark blue) and immature fibrous tissue (pale blue) as well as lobular architecture and small hepatocytes (as characteristic of regeneration). Moreover, the following parameters were analyzed: intercellular space as an area (%); the area of collagen ($\mu m^2/mm^2$); total number of cells/mm^2; total hepatocyte number/mm^2; total hepatocyte nuclei number/mm^2; mononuclear hepatocytes/mm^2; number of multinucleated hepatocytes /mm^2; non-hepatocytes (other) cells number/mm^2.

Intercellular space as an area (%) and the area of collagen ($\mu m^2/mm^2$) were analyzed on the microscopic images with the use of pixel count method powered by software's color threshold function to isolate fibers or intercellular spaces of appropriate color and hue. Such isolation facilitated assessment of the spatial distribution of collagen fibers and intercellular space. Subsequent software pixel counting function was used, and the amount of collagen fibers or intercellular spaces were calculated with appropriate scale as described previously (Tomaszewska et al., 2014; Dobrowolski et al., 2016).

Determination of the content of Cu in liver

A liver sample weighing about 5.0 g with an accuracy of 0.001 g was put into quartz crucibles and burnt at 450°C. The resulting ash was then dissolved in a specified volume of 1M of nitric acid. The content of Cu was determined by means of an Avanta PM flame atomic absorption spectrophotometer manufactured by GBC.

Statistical analysis

All the results are expressed as means ±SD (standard deviation). Differences between the means were tested with the one way ANOVA and post hoc Tukey's test as the correction for multiple comparisons. Normal distribution of data was examined using the W. Shapiro-Wilk test and equality of variance was tested by the Brown-Forsythe test. A P-value of less than 0.05 was considered statistically significant. All statistical analyses were carried out by means of Statistica 12 software (StatSoft, Inc., Tulsa, OK, USA; http://www.statsoft.com).

Results

Food consumption was measured daily in control animals and those treated with Cu, but there were no differences. There also were no differences in weekly water consumption between the groups (Table 2).

The initial and final body weight of the control rats and animals treated with the organic Cu form (regardless of the amount of daily requirement) were similar. There was no effect of Cu supplementation on the absolute or relative liver weight (Table 2).

The Cu liver content of the control rats and animals belonging to groups treated with the organic form of Cu reached similar values (regardless of the daily requirement) (Table 2). The Cu plasma concentration of the control rats and animals sup-

plemented with the organic form of Cu (regardless of the daily requirement) did not differ between one another (Table 3).

Table 2. Mean weekly water and daily feed consumption, initial and final body weight, absolute and relative liver weight, and Cu content in the liver in control rats and animals treated with different levels of chelated Cu

	CONT	OG100	OG75
Water consumption (ml)	85.3±13.8	83.5±12.7	84.5±16.2
Feed consumption (g)	164±14	166±17	169±12
Initial body weight (g)	357±28	351±25	391±15
Final body weight (g)	464±37	476±28	489±15
Absolute liver weight (g)	15.3±0.6	16.4±0.73	16.4±1.1
Relative liver eight (g)	3.29±0.3	3.44±0.7	3.35±0.5
Cu content in liver (µg/g of tissue)	0.0035 ±0.0002	0.0039 ±0.0002	0.0038 ±0.0002

Data given are Mean ± SD.

CONT – the control group received Cu at 100% of daily requirement from sulfate; OG100 – the group received Cu at 100% of daily requirement from Cu-Gly; OG75 – the group received Cu at 75% of daily requirement from Cu-Gly.

Table 3. Basal hematology and blood plasma biochemical parameters of control rats and animals treated with different levels of chelated Cu

	CONT	OG100	OG75
WBC (10^9/L)	3.23±0.49	3.68±1.41	3.87±0.88
RBC (10^{12}/L)	6.80±1.44	7.1±1.1	7.9±0.6
Hb (g/L)	8.65±0.67	9.53±0.73	9.71±1.63
Ht (%)	0.38±0.06	0.36±0.04	0.35±0.22
ALT (U/L)	35±7 a	58±11 b	56±6 b
AST (U/L)	52±6 a	77±6 b	78±4b
AST/ALT ratio	1.48±0.9	1.33±0.5	1.39±0.7
ALP (U/L)	158±9	157±17	158±17
LDH (U/L)	235±75	290±25	295±25
Cu (µmol/L)	25.4±0.7	27.4±0.6	27.7±1.8
Fe (µmol/L)	41.1±3.8	45.3±6.8	45.4±8.2
Zn (µmol/L)	24.2±1.6	25.5±1.8	25.4±1.3
Total protein (g/L)	65.7±3.3	72.1±1.7	71.1±3.7
Glucose (mmol/L)	8.76±0.58	9.8±0.7	9.3±0.4
Total cholesterol (mmol/L)	2.14±0.31	1.98±0.19	1.92±0.25
Triacylglycerol (mmol/L)	1.57±0.18	1.44±0.22	1.46±0.25

Data given are Mean ± SD, a, b – $P<0.05$.

CONT – the control group received Cu at 100% of daily requirement from sulfate; OG100 – the group received Cu at 100% of daily requirement from Cu-Gly; OG75 – the group received Cu at 75% of daily requirement from Cu-Gly.

The basal blood hematology in the control group did not differ from the value obtained in the groups treated with the organic form of Cu at 100% and 75% of daily requirement (Table 3). The ALT activity in rats supplemented with the organic form of Cu (regardless of the amount of Cu-Gly) was higher and differed significantly from the lower activity in the control group. The AST activity in rats supplemented

with the organic form of Cu (regardless of the amount of Cu-Gly) was higher and differed significantly from the lower activity in the control group as well. Although the AST and ALT activities increased in groups supplemented with the organic Cu, the de Ritis coefficient did not drop below one unit or markedly rose, and did not differ from control value. The ALP and LDH enzyme activities in blood serum of the control rats did not differ from the activity in both OG groups. The concentration of Fe and Zn in blood plasma was similar in the control group and groups treated with the organic Cu. There was no difference in the concentration of total protein and glucose between the control group and rats treated with the organic Cu (regardless of the amount of Cu-Gly) (Table 3).

Table 4. Histomorphometry of the jejunum epithelium of control rats and animals treated with different levels of chelated Cu

	CONT	OG100	OG75
Myenteron thickness (μm):			
longitudinal lamina	33±6	24±8	32±8
transversal lamina	59±3a	32±10 b	53±11 a
Submucosa thickness (μm)	55±4 a	29±7 b	35±9 a
Mucosa thickness (μm)	720±98	712±77	703±123
Enterocyte number/100 μm of villus	15±2	14±2	15±3
Villus epithelium thickness (μm)	33±7	35±8	23±3
Villus length (μm)	651±30	618±60	748±131
Villus thickness (μm)	108±5 a	83±10 b	111±5 a
Total number of villi/mm	8±1	6±1	7±1
Crypt depth (μm)	146±12 a	65±14 b	169±38 a
Crypt width (μm)	42±5	34±6	50±7
Active crypt number/mm	8±2	7±3	7±2
Inactive crypt number/mm	9±4	10±4	9±2
Total crypt number/mm	17±4	17±6	15±2
Small intestine absorptive surface (μm^2)	14±3	16±3	13±3

Data given are Mean ± SD; a, b – $P<0.05$.

CONT – the control group received Cu at 100% of daily requirement from sulfate; OG100 – the group received Cu at 100% of daily requirement from Cu-Gly; OG75 – the group received Cu at 75% of daily requirement from Cu-Gly.

The intake of Cu in Cu-Gly form at 100% of daily requirement resulted in the thinner transversal myenteron, narrower villi and submucosa as well as shallower crypts compared with the control group (supplemented with Cu in the inorganic form at 100% of daily requirement) and the group supplemented with Cu amino acid chelate at 75% of daily requirement (Table 4). The administration of Cu in Cu-Gly form at 75% of daily requirement did not influence the histomorphometric parameters of intestinal epithelium.

The Cu supplementation in the organic form (regardless of the amount) did not influence the histomorphometrical parameters of nerve plexus in jejunum compared to the Cu supplementation in the inorganic form (Table 5).

Table 5. Histomorphometry of the nerve plexuses in the jejunum of control rats and animals treated with different levels of chelated Cu

	CONT	OG100	OG75
Auerbach plexus			
area (μm²)	995±1115	445±615	867±1192
perimeter (μm)	175±134	90±66	139±129
mean Feret (μm)	52±39	27±19	42±38
min diameter (μm)	11±6	11±5	15±5
mean diameter (μm)	23±12	19±9	25±13
sphericity	0.09±0.14	0.25±0.21	0.27±0.24
Meissner plexus			
area (μm²)	534±298	360±192	409±249
perimeter (μm)	97±33	84±27	84±33
mean Feret (μm)	29±9	24±7	25±9
min diameter (μm)	18±7	13±3	16±4
mean diameter (μm)	24±7	20±5	21±6
sphericity	0.38±0.20	0.38±0.21	0.40±0.21

Data given are Mean ± SD; a, b – $P<0.05$.

CONT – the control group received Cu at 100% of daily requirement from sulfate; OG100 – the group received Cu at 100% of daily requirement from Cu-Gly; OG75 – the group received Cu at 75% of daily requirement from Cu-Gly.

Table 6. Histomorphometrical parameters of liver tissue of control rats and treated with different levels of chelated Cu

	CONT	OG100	OG75
Intercellular space (%)	5±3 a	10±4 b	9±3 b
Collagen area (μm²/mm²)	33.29±17.64 a	57.39±22.71 b	56.58±27.89 b
Total cell number/mm²	2618±482	2895±1000	2210±394
Total hepatocyte number/mm²	1941±440	2261±979	1611±359
Total hepatocyte nuclei number/mm²	2015±482	2347±1035	1670±385
Mononuclear hepatocyte number/mm²	1870±409	2175±925	1553±334
Multinuclear hepatocyte number/mm²	73±42	86±59	58±36
Other cells/mm²	676±122	633±146	598±149

Data given are Mean ± SD; a, b – $P<0.05$.

CONT – the control group received Cu at 100% of daily requirement from sulfate; OG100 – the group received Cu at 100% of daily requirement from Cu-Gly; OG75 – the group received Cu at 75% of daily requirement from Cu-Gly.

Microscopic assessment of liver structure in rats supplemented with Cu in the organic form (regardless of daily requirement) showed no marked differences in portal triads and terminal hepatic venules distribution in the tissue compared to the control supplemented with Cu sulfate. Moreover, Cu supplementation (irrespective of the type of Cu) did not change general lobular architecture on the level of low magnification microscopic observation. The histological examination of the control liver tissue showed normal architecture hepatocytes, which were large in size, hexagonal in shape with more or less centrally located nuclei and homogenous cytoplasm. However histomorphometric analysis showed the increase of collagen amount and

intracellular space in the group supplemented with Cu amino acid regardless of the percentage of daily requirement (Table 6).

Discussion

The Cu content in the diet differs markedly because foodstuffs contain varied amounts thereof. Hence, the Cu intake from the environment is limited in humans and animals. Dietary supplements containing Cu are the main sources of this trace element. Thus, it is necessary to examine the effects of the supplementation of different Cu forms on histomorphometric changes in liver and intestine when 100% of the daily requirement is met. Additionally, in the current study, one of the diets supplemented with organic Cu met 75% of the daily requirement.

The present results showed that there was no direct effect of the dietary Cu content in the different sources on the feed intake in adult rats. In addition, Cu supplementation at the experimentally lowered level in the glycine chelate form did not affect the body weight and liver weight. These results are compatible with the effect observed in a previous study performed on adolescent rats supplemented with Cu in the glycine chelate form at 25%, 50%, and 75% of the required amount (Tomaszewska et al., 2014). Another study performed on broiler chickens has proved the beneficial effect of a Gly-Cu additive in the diet on the growth and development as well as absence of a negative action on the feed to gain ratio and carcass quality (Kwiecień et al., 2015 a). However, a study on pigs has indicated that the average daily gain tends to be higher in pigs fed with Cu with a lysine complex than in pigs fed with $CuSO_4$ (Apgar and Kornegay, 1996). On the other hand, our result was in contrast to others, which showed that Cu deficient diet leads to a reduction in body weight because Cu is essential for normal growth and development (Megahed et al., 2013).

Most dietary Cu passes through the liver, where it can be used for protein and energy production, and is then excreted by the biliary route (Linder and Hazegh-Azam, 1996). Data from rat studies indicate that, in the range of normal intakes, there is adaptation of absorption relative to the need (Linder and Hazegh-Azam, 1996). The mechanism by which Cu enters hepatocytes from albumins contains a transporter system like a complex of Cu-histidine in hepatocytes (Linder and Hazegh-Azam, 1996). Thus, Cu homeostasis is mainly maintained by the efforts of the intestine and liver. The liver is the central organ of Cu metabolism as well as the most sensitive organ to Cu deficiency (Linder and Hazegh-Azam, 1996). However, an interaction related to an increase in liver Cu concentrations was noted when ewes were fed increasing dietary Cu from $CuSO_4$ but not when they were fed Cu proteinate diets (Eckert et al., 1999).

The current study indicated that all the adult rats (regardless of the Cu amount in the diet) had a comparable concentration of iron, zinc, and total protein in blood serum, and their basal hematological parameters were not changed. Moreover, in our animals, there was no Cu deposition in the liver, because there was no difference in the liver or plasma Cu concentration between the control group (supplemented

with Cu sulfate at 100% of daily requirement) and the groups supplemented with the glycine chelate of Cu (regardless of the Cu amount in the diet). This may indicate that there was no excessive Cu absorption and storage in the adult rats. These results are in agreement with a previous study performed on adolescent rats also supplemented with inorganic (sulfate) and organic (glycine chelate) forms of Cu at 100% of daily requirement (Tomaszewska et al., 2014). However, in contrast to an earlier study, the biochemical analysis performed in this study showed that the adult rats had no elevated LDH and ALP activity. Lactate dehydrogenase is clinically the most important of several enzymes occurring in blood serum. It is present in each tissue in the cellular cytoplasm, and it is known that the LDH fraction in the liver tissue is reduced and released into the bloodstream after cell damage (Dobryszczycka and Owczarek, 1981). The current study showed that the diet containing the organic form of Cu (amino acid chelate) did not enhance the activity of LDH, but elevated the activities of ALT and AST (regardless of the Cu amount in the diet). Although no increase in the de Ritis coefficient was observed in our adult rats, the elevated AST and ALT activities can suggest the alteration in liver tissue.

Gastrointestinal effects have also been reported in other animal studies. Wang et al. (2007) and Kwiecień et al. (2015 a) have shown that the form of Cu additives significantly affects the Cu content in the liver of broiler chickens. Similarly, other studies have indicated that the Cu content in the liver of turkeys is higher when Cu is added in the form of a chelate (Makarski, 2002). In addition, a slightly higher concentration of Ca, Zn, and Fe has been noted in birds' liver (Makarski et al., 2009 b). On the other hand, Bao et al. (2007) did not observe any significant alteration in the Cu liver content in chickens supplemented with an organic and inorganic form. Furthermore, a study on chickens has shown that the organic form enhances the activity of ALT, AST, and LDH in chicken blood compared to the activity of these enzymes at administration of the inorganic form (Kwiecień et al., 2015 a). Especially, an increase in the LDH concentration might suggest damage to birds' liver. Similarly, Makarski et al. (2009 a) have observed higher activity in the case of the use of lysine and a Cu chelate. The available animal data report that one of the most commonly adverse health effects of copper is hyperplasia mucosa following ingestion of copper sulfate in the diet in rats and mice exposed to 44 and 197 mg Cu/kg/day, respectively. No gastrointestinal effects were observed in rats and mice exposed to 23 or 92 mg Cu/kg/day for 14 days or in rats and mice exposed to 16 or 126 mg Cu/kg/day for 13 weeks. Additionally, no gastrointestinal effects were observed in rats and mice exposed to 29 or 24 mg Cu/kg/day as copper sulfate in drinking water (Hebert, 1993).

A previous study has shown that dietary supplementation of a Cu amino acid chelate affects the liver structure in adolescent rats supplemented with a glycine chelate of Cu at 100% and 75% of daily requirement (Tomaszewska et al., 2014). This is in some contrast with the results from our adult rats supplemented with the organic form of Cu, which did not affect the liver tissue as negatively as earlier in adolescent individuals. It is worth mentioning that the same dietary organic Cu concentrations and exposure time were used in these two studies, but the divergence might be due to the different age of the rats. Another study has also shown that young copper-loaded

rats accumulate more hepatic copper, had more severe liver changes (substantial liver injury), and had higher serum liver enzyme activities than adult rats (Fuentealba et al., 2000). Moreover, numerous studies in animal models and human volunteers have shown a link between Cu deficiency and altered lipid metabolism associated with non-alcoholic fatty-liver disease and non-alcoholic hepatitis (Klevay et al., 1984; Salama et al., 2007; Aigner et al., 2010). However, our previous study performed on adolescent rats shows that Cu given in the diet in a Cu-glycine complex, irrespective of its dose, did not alter glucose and lipid metabolism (Tomaszewska et al., 2015). Thus, the concentrations of glucose, cholesterol, and triacylglycerol were assessed in the present study. The lack of differences in the glucose concentration between the investigated groups may indicate that our adult rats did not exhibit deficient or defective activity of enzymes responsible for metabolic transformation of glycogen in the body and abnormal accumulation resulting in dysfunction of the liver or heart and kidneys, as described in the glycogen storage disease, i.e. glycogenosis. Moreover, the Cu chelate given at 75% of daily triacylglycerol in the diet to our adult rats did not change the concentration of total cholesterol or triacylglycerol. It is known that in a copper deficient state, the serum cholesterol level is elevated because of more rapid synthesis and clearance thereof into blood plasma and a limited cholesterol pool for excretion as biliary steroids (Allen and Klevay, 1978). On the other hand, it is known that a copper-dependent enzyme lysyl oxidase is involved in cross-linking of collagen and elastin. Thus, elevated plasma cholesterol in the Cu-deficient state might lead to cardiac hypertrophy or hemorrhage linked with deformation of the aorta and loss of elasticity by arteries (Allen and Klevay, 1978).

It is known that the structure of the intestinal mucosa gives some information on gut health, e.g. shortening of the villus decreases the surface area for nutrient absorption. The crypt is an area where stem cells divide to permit renewal of the villus; a large crypt indicates fast tissue turnover and a high demand for new tissue (Xia et al., 2004). Different factors occurring in the digesta can cause relatively rapid changes in the intestinal mucosa due to the close proximity of the intestinal contents and the mucosal surface. A study performed on weanling pigs showed that the duodenal villus height was reduced in a group supplemented with Cu sulfate, but inorganic salt was given in higher amounts (225 mg of Cu/kg of a diet) than the minimum daily dose by American standards, which recommend 6–125 ppm depending on the production cycle (NRC, 1998; Fry et al., 2012). Therefore, in this study, we decided to use two copper sources contemporaneously, lowering the amount of dietary Cu in relation to the daily requirement defined as providing a minimum of the trace element in order to cover the required amount and maintain normal homeostasis for a 24-hour period in adult rats. Moreover, Cu supplementation in the broiler diet significantly influenced the morphology of the intestinal tract; it depressed the height of villi and significantly thickened the muscular layer in the duodenum, but this diet was supplemented with Cu in a dose higher than 250 mg/kg (Chiou et al., 1999). Another study showed that rats fed with diet supplemented with 80 mg/kg body weight of $CuSO_4$ had no significant effects of Cu on the villus height and crypt depth of small intestinal mucosa (Han et al., 2012). Furthermore, Cu deficiency induced in Friesian cattle fed with diet containing less than 1 mg Cu/kg resulted in lesions of the small intes-

tine (Millsa et al., 1976). Moreover, our earlier study performed on adolescent rats indicated that Cu given in the organic form meeting 100% of daily requirement in adolescent rats depressed the number and height of enterocytes, but did not affect the enteric nervous system, and organic Cu given in an amount lowered to 75% or 50% of daily requirement did not influence the morphology of enterocytes (Tomaszewska et al., 2015). On the other hand, an increase of the villus height was observed in the small intestinal mucosa of chicks supplemented with a copper-bearing montmorillonite complex or in rats treated with copper-loaded chitosan nanoparticles (Xia et al., 2004; Han et al., 2012).

No studies conducted so far have provided a detailed morphological analysis of the small intestine of adult rats administered with diet containing different Cu sources. Based on the histomorphometric analysis of the jejunum, the current study indicated that Cu given in the chelate form covering 100% of the daily requirement decreased the muscular and submucosa layers as well as the crypt depth without an influence on the innervation of the jejunum. In turn, Cu given in the amino acid form in the lowered amount in relation to the daily requirement did not influence the intestine morphology in the adult rats.

Potentially, the use of chelated minerals with higher bioavailability can allow reduction of the supplemented amount of Cu and waste from unassimilated minerals. Amino acid chelates have significantly higher absorption rates from the intestine compared to soluble inorganic metal salts, but the supplementation of the diet is often difficult and not economically viable (Ashmead et al., 1985; Reyes, 1996; Andersen, 2004).

No studies conducted so far have provided a detailed morphological analysis of the small intestine of adult rats administered with diet containing different Cu forms. Dietary Cu given to adult rats in inorganic form as well as in the amino acid form covering 75% of the daily requirement appears to be less harmful with regard to the intestinal epithelium and liver. However, further studies are needed to clarify the mechanism of the influence of the amino acid chelate form of trace elements on liver and intestinal epithelium morphology.

Conflict of interest

There are no known conflicts. Financial support for this work does no influence its outcome. The manuscript has been read and approved by all named authors and there are no other persons who satisfied the criteria for authorship but are not listed. The order of authors listed in the manuscript has been approved by all authors.

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