Ann. Anim. Sci., Vol. 17, No. 4 (2017) 1089–1105 DOI: 10.1515/aoas-2017-0022

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DOSE-DEPENDENT INFLUENCE OF DIETARY CU-GLYCINE COMPLEX ON BONE AND HYALINE CARTILAGE DEVELOPMENT IN ADOLESCENT RATS*

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Abstract

Administration of the amino acid copper (Cu) complex ensures higher Cu bioavailability through enhanced absorption from intestine and decreases the dietary Cu level, compared to the recommended Cu dose. The objective of this study was to investigate the effect of Cu-low diet on the bone development in adolescent rats. Male rats at the age of 6 weeks were used in the 12-week experiment. The control diet provided the required Cu level from sulfate (S-Cu) and other diets were supplemented with Cu as a glycine complex (Cu-Gly) at 25%, 50%, 75%, and 100% of daily requirement. After the 12-week treatment, rats from the Cu-Gly100 group were heavier, compared to the other groups. The copper and calcium plasma and bone concentrations of the rats in the groups treated with the organic form of Cu (irrespective of its dose) was similar to the control values noted in the rats administered with S-Cu. A decrease in the femur weight and length was observed in the Cu-Gly75 and Cu-Gly50 groups. Cu-Gly increased the cross section area, mean relative wall thickness, and cortical index only in the Cu-Gly75 group. A decrease in the ultimate strength, elastic stress, and ultimate stress was noted in the Cu-Gly100 and Cu-Gly75 groups. In the Cu-Gly50 group, a decrease in the ultimate stress and an increase in the maximal elastic strength and bending moment were noted. Adolescent rats treated with Cu-Gly at a Cu-deficient level exhibited a dose-dependent strongly osteoporotic cancellous bone. Lower proteoglycan content was found in groups fed the Cu-low diet. In the control rats supplemented with S-Cu, there was no evident gradient in safranin O staining. It is difficult to indicate which dose of the Cu-Gly complex among the investigated Cu-poor diet exerted a positive effect on bone metabolism. It appears that the use of this Cu-Gly complex at a significantly reduced dose than S-Cu at the recommended dose did not inhibit the development of bone and hyaline cartilage in adolescent rats.

Key words*:* copper, copper-glycine complex, bone histomorphometry, mechanical testing, adolescent rat

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*Work financed from statutory research of Department of Animal Physiology University of Life Sciences in Lublin no. WKF/DS/1 and statutory research of Department of Comparative Anatomy and Anthropology of Maria Curie-Skłodowska University no. BS-01-1200-0000.

Appropriate nutrition is a basic health requirement for adolescents to express their genetic potential properly in terms of growth and development (Urbano et al., 2002). Due to the intensive growth rate during adolescence, the demand for some minerals is highly important. An imbalanced diet during adolescence can substantially delay growth and increase the risk of some chronic diseases such as osteopenia or osteoporosis in later life. As an essential trace element, copper (Cu) is necessary for adolescent growth because it is an important cofactor for several enzyme systems and hemoglobin synthesis (Urbano et al., 2002). Young individuals have a substantially higher level of Cu per unit body weight than adults. It is the third most abundant essential trace element in animals and humans, besides iron and zinc. Cu deficiency is associated with anemia, neutropenia, cardiac disorders, and skeletal abnormalities (Mesías et al., 2012). It is known that Cu takes part in bone metabolism and turnover and influences the development of the skeleton in humans and animals as an essential cofactor required for the activity of lysyl oxidase (Rodríguez et al., 2002). This Cu-dependent enzyme is targeted exclusively at extracellular collagen molecules, regulating their total enzymatic cross-link formation in connective tissue. Moreover, lysyl oxidase mediates the final step in the biosynthesis of elastin and normalizes the deposition of calcium and phosphorus in bones (Linder and Hazegh-Azam, 1996). Studies on Cu supplementation in humans indicate that Cu insufficiency leads to bone loss (osteopenia or osteoporosis), which causes a decrease in mechanical endurance and consequent fractures (Nielsen and Milne, 2004). This is caused by decreased function of osteoblasts (bone tissue forming cells), while the action of osteoclasts (bone tissue resorbing cells) remains unaffected (Rodríguez et al., 2002). Thus, inadequate Cu dietary intake in adolescence can be an important factor in the etiology of bone loss, osteoporosis, or even osteoarthritis developing in the adult life (Palacios, 2006). Moreover, it is commonly known that the achievement of higher peak bone mass during adolescence protects against postmenopausal osteoporosis (Nielsen and Milne, 2004).

Organic sources such as those offered in an amino acid complex ensure higher bioavailability of Cu, because absorption thereof from the small intestine is enhanced by amino acids (Männer et al., 2006). This seems to be related to the fact that cationic trace minerals should be chelated by proteins at the brush border of the cell membrane prior to absorption in the intestine, thus slowing down the process. No additional chelation of amino acid chelates is required and the membrane transport is more rapid (Linder and Hazegh-Azam, l996). Studies in humans and animals indicate that the absorption is regulated by the nutritional status and depends on the chemical form in which the microelement is present (Świątkiewicz et al., 2001; Tomaszewska et al., 2014; Tomaszewska et al., 2016 b; Tomaszewska et al., 2016 c; Tomaszewska et al., 2017; Ognik et al., 2016).

The relationship between the dietary status of Cu and bone health and cartilage has not been extensively studied. Additionally, studies in growing subjects are limited. Given the importance of Cu in bone metabolism during adolescence, the objective of this study was to investigate the effect of administration of the amino acid Cu complex in male growing rats fed Cu-low diet, which ensured higher Cu bioavailability through enhanced absorption from intestine, compared to the recommended

Cu dose. A possible influence exerted by the intake of the two different chemical forms on bone metabolism was also studied.

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 in Lublin, Poland. The rats were maintained in an animal house according to the guidelines formulated by the committee. All efforts were made to minimize the number of animals used as well as their suffering.

Animals, breeding, and experimental design

Clinically healthy male adolescent Wistar rats at the age of 6 weeks at the start of the experiment (excluding weekly acclimatization) were used in the 12-week experiment. The animals were kept individually in Macrolon cages at 21±1°C, 55% humidity, and 12-hour light and dark cycles. The rats were randomly divided into the control and 4 experimental groups (each n=12). All rats had free access to distilled water (no Cu, thus it was considered important with respect to the Cu intake) and fed *ad libitum*. The basal diet included (LSM, Agropol S.J., Motycz, Poland) crude protein min. 14.5%, carbohydrates 64%, crude fat min. 1.5%, crude fiber min. 5%, ash 10%, calcium min. 1.10%, and inorganic phosphorus min. 0.70%. The Cu level in the plants contained in the basal diet was not determined. The content of vitamin and mineral premixes of the diet is presented in Table 1. The control group received standard diet with addition of the required Cu level for rats in the inorganic S-Cu form (S-Cu group; 5 mg/kg of body weight per day from sulfate ($CuSO_4$)) (Megahed et al., 2014). The rats from the experimental groups received the same standard diet with addition of 100%, 75%, 50%, and 25% of Cu daily requirement in an organic form (5; 3.75; 2.5, and 1.25 mg/kg of body weight per day from the Cu-glycine complex) in the Cu-Gly100, Cu-Gly75, Cu-Gly50 and Cu-Gly25 groups, respectively. Water and feed consumption levels were measured weekly. At the end of the experiment, the rats were fasted for 24 hours and euthanized one by one with carbon dioxide inhalation and by dislocation of the spine.

Plasma biochemical analyses

Blood samples were collected twice, at the 6th week and at the end of the study, by standard cardiac puncture. Plasma for biochemical analyses was separated immediately by centrifugation and stored at –25°C. Colorimetric analysis was used to determine plasma concentrations of calcium (Ca), copper (Cu), iron (Fe), and zinc (Zn) by means of a Metrolab 2300 GL unit (Metrolab SA, Buenos Aires, Argentina) and using ready-made sets (BioMaxima, Lublin, Poland). Additionally, the plasma concentration of phosphorus (P) was determined with the same method at the end of the study.

Table 1. Composition of vitamin and mineral premixes of the diet (per kilogram dry matter) given to the 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
Vitamin E (mg)	4 964
Menadione sodium bisulfite (mg)	300
Riboflavin (mg)	600
Pyridoxine HCL (mg)	60
Cyanocobalamin (mg)	1.2

Bone collection and analysis

Left femora collected from each animal were measured and weighed after removal of soft tissues. Each bone was wrapped in gauze soaked in isotonic saline and stored at –25ºC for further analysis. Geometric properties: cross-section area (A), mean relative wall thickness (MRWT), and cortical index (CI) were estimated in the mid-diaphyseal cross-sections as described previously (Tomaszewska et al., 2016 a; Muszyński et al., 2017).

The mechanical properties of the femur (ultimate strength and maximum elastic strength) were determined as described previously (Tomaszewska et al., 2015; Muszyński et al., 2017). The material properties of the bone were also calculated based on measured geometric and mechanical traits as described previously (Muszyński et al., 2017).

The samples of the distal end of each femur were subjected to histology as described previously (Dobrowolski et al., 2016). Two methods of staining were used: the Goldner's trichrome to assess the morphology of the growth plate and articular cartilage and the safranin O staining to visualize cartilage proteoglycans (Dobrowolski et al., 2016). Briefly, sagittal sections in the middle of the lateral condyle of each femur were cut strictly following the method and equipment described previously (Dobrowolski et al., 2016). Safranin O staining was applied in the visual assessment using the Mankin histological and histochemical grading system for evaluation of articular cartilage (Kadri et al., 2008).

The thickness of the following zones: reserve (I), proliferation (II), hypertrophy (III), and ossification (IV) was measured at four sites along the growth plate cartilage and an average was calculated as described previously (Hochberg, 2002; Tomaszewska et al., 2016 b). Similarly, the thickness of the main zones of the articular cartilage, i.e. horizontal (superficial surface, I), transitional (II), radial (III), and calcified zone (IV) was measured as described previously (Hochberg, 2002; Tomaszewska et al., 2016 b).

The bone volume (BV), tissue volume (TV), relative bone volume (BV/TV%), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and trabecular number

(Tb.N) were measured as described previously (Dobrowolski et al., 2016; Tomaszewska et al., 2016 b).

After evaluating the strength and structural properties, the bones were defatted, dried to constant mass, and finally mineralized in a muffle furnace at 600°C (AOAC, 2000; Tomaszewska et al., 2016 b). The content of mineral components (Ca, P, Cu) in bones was determined by atomic absorption spectrometry using a Unicam 939/959 apparatus. The percentage of bone ash and the content of Ca, P, and Cu in the bone were calculated as the content of these components in crude ash.

Statistical analysis

All the results are expressed as means ± SD (standard deviation). Differences between the means were tested with 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 the equality of variance was tested with the Brown-Forsythe test. A P-value 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

Initial and final body mass, weekly water and feed consumption

The initial body weight of the control rats and those treated with the organic Cu form (regardless of the percent of daily requirement) were similar (Table 2). At the end of the study, i.e. after 12 weeks of the treatment, only the rats from the Cu-Gly75 group were heavier and had the highest daily weight gain compared to the control group (Table 2). The highest weekly water and feed consumption levels were noted in the Cu-Gly50 and Cu-Gly25 groups compared to the control group (Table 2).

Table 2. Initial and final body weight, weekly consumption of water and feed, and daily weight gain in the control group and in rats treated with different levels of Cu in the Cu-Gly complex

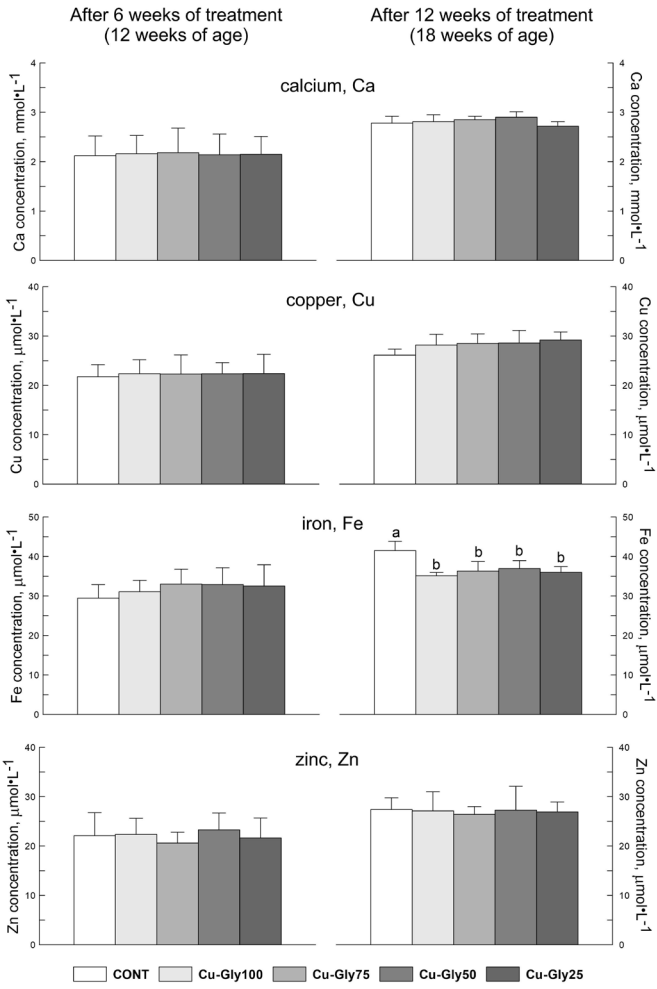
Group	n	Body weight (g)		Daily weight gain (g)	Water consumption (ml)	Feed consumption (g)
		Initial body weight (g)	Final body weight (g)			
CONT	12	223.7±2.4	446.2±36.9 a	2.9±0.5 a	66.6±42.6 a	157.5±13.9 a
Cu-Gly100	12	213.5±2.6	447.4±28.3 ab	3.1±0. a	81.7±14.7 ab	172.4±17.3 abc
Cu-Gly75	12	217.9±6.1	501.1±5.8 b	3.4±0.1 b	98.3±11.8 ab	182.4±10.5 bc
Cu-Gly50	12	223.7±10.3	491.1±14.2 ab	3.2±0.3 a	101.2±9.2 b	182.2±5.3 bc
Cu-Gly25	12	222.3±14.2	483.0±4.5 ab	3.1±0.2 a	100.2±16.6 b	189.3±16.6 c

a, b, c – mean values in the columns with different letters differ significantly at $P<0.05$; Data given are Mean ± SD (standard deviation); SEM – standard error of the mean.

CONT – the control group received Cu at 100% of daily requirement from sulfate; Cu-Gly100 – the group received Cu at 100% of daily requirement from Cu-Gly; Cu-Gly75 – the group received Cu at 75% of daily requirement from Cu-Gly; Cu-Gly50 – the group received Cu at 50% of daily requirement from Cu-Gly; Cu-Gly25 – the group received Cu at 25% of daily requirement from Cu-Gly.

The content of Ca, total P, Fe, Zn, and Cu in blood plasma

The Cu plasma concentration in the rat groups treated with the organic form of Cu (irrespective of its dose) for 6 weeks reached similar values as those in the control rats administered with S-Cu (Figure 1). In addition, the Ca, Fe, and Zn plasma concentrations were similar in the control rats and animals supplemented with the organic form of Cu (regardless of the percent of daily requirement) and did not differ between each other (Figure 1).



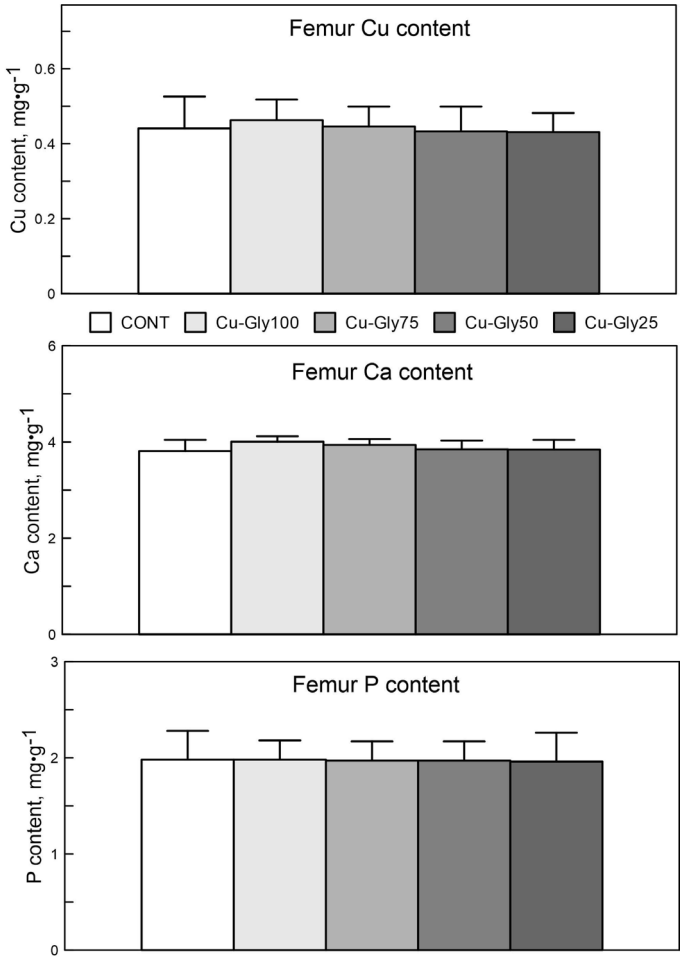
Data given are Mean ± SD; * P<0.05.
CONT – the control group received Cu at 100% of daily requirement from sulfate; Cu-Gly100 – the group received Cu at 100% of daily requirement from Cu-Gly; Cu-Gly75 – the group received Cu at 75% of daily requirement from Cu-Gly; Cu-Gly50 – the group received Cu at 50% of daily requirement from Cu-Gly; Cu-Gly25 – the group received Cu at 25% of daily requirement from Cu-Gly.

Figure 1. Plasma concentration of calcium (Ca), copper (Cu), iron (Fe), and zinc (Zn) in blood plasma in the control rats and in rats treated with different levels of Cu-Gly at the age of 12 and 18 weeks

At the end of the experiment, i.e. after 12 weeks, the Cu, Zn, and Ca plasma concentrations did not differ among the groups (Figure 1). In turn, the Fe level was lower in the rats supplemented with the Cu-Gly form (irrespective of its dose), compared to the control group administered with S-Cu (Figure 1). Moreover, the P plasma concentrations did not differ among the groups and reached the values of 2.55±0.1 mmolL^{-1}, 2.54±0.5 mmolL^{-1}, 2.54±0.3 mmolL^{-1}, 2.53±0.2 mmolL^{-1}, and 2.52±0.4 mmolL^{-1} in the control, Cu-Gly100, Cu-Gly75, Cu-Gly50, and Cu-Gly25, respectively.

The content of P, Ca, and Cu in bone

The bone Cu, P, and Ca content did not differ among the groups (Figure 2).



Data given are Mean ± SD; * P<0.05. Description of the groups as in Figure 1.

Figure 2. Bone content of calcium (Ca) and copper (Cu) in control 18-week-old rats and in rats treated with different levels of Cu-Gly

Table 3. Physical, mechanical, and geometric properties of femur obtained from 18-week-old rats in the control group (S-Cu treated) and in rats treated with different levels of Cu-Gly

Item	Group					SEM	P-value
	CONT (n=12)	Cu-Gly100 (n=12)	Cu-Gly75 (n=12)	Cu-Gly50 (n=12)	Cu-Gly25 (n=12)		
Bone general properties							
Bone weight (g)	1.22±0.01 a	1.11±0.07 ab	1.04±0.12 b	1.08±0.11 b	1.15±0.04 ab	0.023	<0.001
Bone length (mm)	39.00±0.89 a	36.33±0.82 bc	35.00±0.63 c	37.33±0.52 c	36.83±0.98 b	0.277	<0.001
Mass/length ratio	0.031±0.001	0.031±0.001	0.029±0.003	0.029±0.003	0.031±0.002	0.001	0.993
Bone geometrical properties							
Horizontal internal diameter h (mm)	2.59±0.15 a	2.00±0.29 bc	1.77±0.17 c	2.31±0.23 ab	2.41±0.15 a	0.064	<0.001
Horizontal external diameter H (mm)	4.29±0.12 ab	4.38±0.15 ab	4.57±0.06 b	4.42±0.29 ab	4.09±0.26 a	0.044	0.005
Vertical internal diameter b (mm)	1.58±0.06 a	2.40±0.98 b	1.61±0.08 a	1.65±0.20 ab	1.64±0.10 ab	0.096	0.019
Vertical external diameter B (mm)	3.31±0.06 a	2.59±0.72 b	3.33±0.14 a	3.41±0.21 a	3.31±0.16 a	0.092	<0.001
Cross section area A (mm^2)	8.02±0.28 a	8.30±1.14 ab	9.63±0.81 b	8.63±0.79 ab	7.57±1.18 a	0.190	0.003
Mean relative wall thickness	0.89±0.03 a	1.06±0.32 ab	1.31±0.09 b	1.01±0.09 a	0.85±0.10 a	0.041	<0.001
Cortical index CI (%)	46.50±1.06 a	50.30±7.80 ab	56.10±1.38 b	49.95±3.98 ab	45.00±2.88 a	1.001	0.001
Midshaft volume (mm^3)	1.24±0.05 ab	0.72±0.68 a	1.36±0.07 b	1.32±0.13 b	1.11±0.12 ab	0.069	0.012
Moment of inertia Ix (mm^4)	7.15±0.42 ab	6.75±0.57 a	7.98±0.61 ab	8.67±1.59 b	7.02±1.52 ab	0.224	0.026
Index of gyration Rg (mm)	0.94±0.01 ab	0.90±0.03 a	0.91±0.04 a	1.00±0.06 b	0.96±0.03 b	0.009	0.001
Bone mechanical properties							
Ultimate strength (N)	149.50±4.23 a	126.17±7.49 bc	118.33±11.69 c	143.83±16.89 ab	138.83±11.46 ab	2.854	<0.001
Maximal elastic strength (N)	90.00±13.04 ab	75.00±1.79 ab	71.67±7.53 a	100.83±32.93 b	69.33±2.42 a	3.555	0.010
Elastic stress (MPa)	80.77±5.49 a	51.54±10.26 b	52.34±4.02 b	74.05±20.45 a	62.90±15.13 ab	3.041	0.001
Ultimate stress (MPa)	135.28±8.36 a	85.84±12.05 d	86.49±6.99 cd	107.10±13.39 bc	123.66±18.10 ab	4.223	<0.001
Bending moment (N·m)	3.50±0.44 ab	2.73±0.05 a	2.51±0.27 a	3.77±1.23 b	2.55±0.14 a	0.140	0.002

a, b, c – mean values in the rows with different letters differ significantly at $P<0.05$; Data given are Mean ± SD (standard deviation); SEM – standard error of the mean.

CONT – the control group received Cu at 100% of daily requirement from sulfate; Cu-Gly100 – the group received Cu at 100% of daily requirement from Cu-Gly; Cu-Gly75 – the group received Cu at 75% of daily requirement from Cu-Gly; Cu-Gly50 – the group received Cu at 50% of daily requirement from Cu-Gly; Cu-Gly25 – the group received Cu at 25% of daily requirement from Cu-Gly.

Table 4. Histomorphometrical parameters of trabeculae of cancellous bone in femur obtained from 18-week-old rats in the control group (S-Cu treated) and in rats treated with different levels of Cu-Gly

Item	Group					SEM	P-value
	CONT (n=12)	Cu-Gly100 (n=12)	Cu-Gly75 (n=12)	Cu-Gly50 (n=12)	Cu-Gly25 (n=12)		
Epiphysis							
BV/TV (%)	50.23±9.24 a	46.68±0.92 a	31.34±2.40 b	44.09±2.12 a	67.58±3.50 c	2.309	<0.001
TbTh mean (µm)	38.61±2.65 a	35.55±0.44 ab	31.29±1.94 b	41.83±8.33 a	85.22±2.83 c	3.721	<0.001
TbTh max (µm)	103.52±8.86 a	99.76±2.87 a	99.75±20.86 a	94.79±9.44 a	236.21±38.00 b	10.741	<0.001
TbSp mean (µm)	69.68±12.53 a	98.91±7.24 c	123.53±10.05 d	101.12±8.18 c	34.19±9.07 b	5.952	<0.001
TbSp max (µm)	197.40±32.59 ab	220.62±10.59 b	293.42±53.92 c	321.69±45.71 c	137.54±42.94 a	13.740	<0.001
TbN /mm of bone	12.98±1.89 a	13.13±0.14 a	10.03±0.73 b	10.75±1.32 bc	7.93±0.26 c	0.275	0.049
Metaphysis							
BV/TV (%)	44.98±3.12 a	38.91±1.09 b	47.36±1.92 a	46.55±5.14 a	44.18±4.77 ab	0.818	0.004
TbTh mean (µm)	69.37±11.29 a	47.15±1.13 b	40.69±2.98 b	63.09±10.85 a	68.93±7.59 a	2.563	<0.001
TbTh max (µm)	139.06±21.30 ab	100.71±6.59 a	124.84±55.47 ab	158.42±7.50 b	154.73±8.95 b	6.061	0.007
TbSp mean (µm)	142.53±9.21 a	149.85±6.02 a	106.82±12.23 b	184.23±22.56 d	75.91±3.51 c	7.238	<0.001
TbSp max (µm)	236.60±15.05 a	305.52±43.15 a	252.69±52.18 a	449.98±123.51 b	250.00±39.24 a	18.436	<0.001
TbN/mm of bone	6.65±1.37	8.26±0.39	8.69±0.92	7.68±2.13	6.41±35	0.385	0.983

a, b, c – mean values in the rows with different letters differ significantly at $P<0.05$; Data given are Mean ± SD (standard deviation); SEM – standard error of the mean.

CONT – the control group received Cu at 100% of daily requirement from sulfate; Cu-Gly100 – the group received Cu at 100% of daily requirement from Cu-Gly; Cu-Gly75 – the group received Cu at 75% of daily requirement from Cu-Gly; Cu-Gly50 – the group received Cu at 50% of daily requirement from Cu-Gly; Cu-Gly25 – the group received Cu at 25% of daily requirement from Cu-Gly.

Bone morphology, geometry, and mechanical properties

The bone weight decreased in the Cu-Gly75 and Cu-Gly50 groups compared to the other groups (Table 3). The intake of Cu in the Cu-Gly form, irrespective of its dose, decreased the femur length, compared to the control group (S-Cu), and did not influence the mass/length ratio. However, 3 of the 4 measured diameters (B, b, h) were altered in the Cu-Gly100 group; the horizontal internal diameter (h) decreased and the horizontal external diameter (H) increased in the Cu-Gly75, compared to the control group (S-Cu) and the other Cu-Gly supplemented groups. Moreover, the cross section area, mean relative wall thickness, and cortical index increased in the Cu-Gly75 group (Table 3). Furthermore, an increase in the midshaft volume was noted in the Cu-Gly75 and Cu-Gly50 groups. An increase in the moment of inertia was observed in the Cu-Gly50, and the index of gyration was higher in the Cu-Gly50 and Cu-Gly25 groups (Table 3).

The ultimate strength as well as elastic and ultimate stresses decreased in the Cu-Gly100 and Cu-Gly75 groups; the ultimate stress decreased but the maximal elastic strength and bending moment increased in the Cu-Gly50 group (Table 3).

Bone histomorphometry

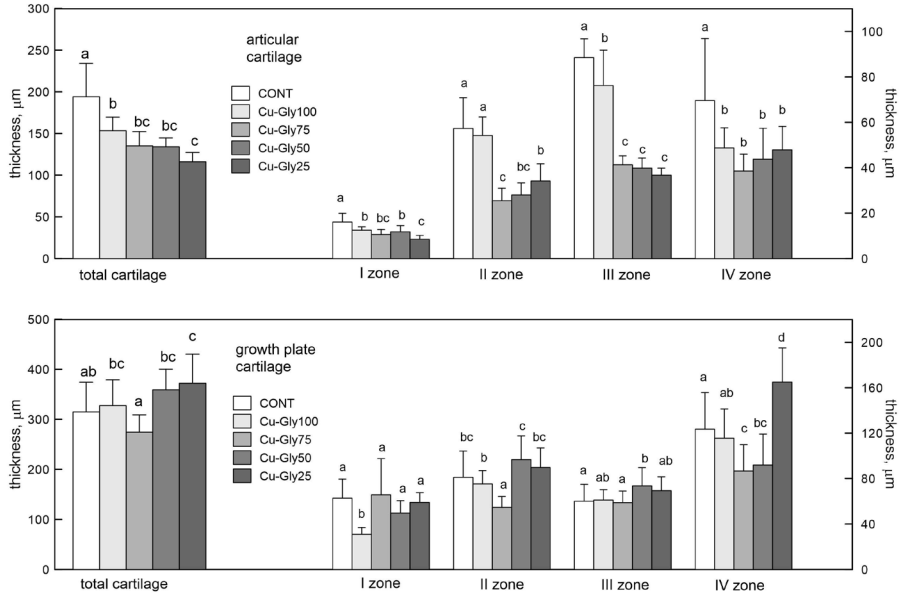
Microscopic assessment of cancellous bone in the Cu-Gly25 rats showed a significant increase in the real bone volume and trabecular thickness and a decrease in the trabecular space in epiphysis, compared to the control group supplemented with Cu sulfate and the other Cu-Gly groups (Table 4). Moreover, the real bone volume and mean trabecular thickness in epiphysis in the Cu-Gly75 group decreased, compared to other groups. Moreover, there was an increase of trabecular space in the Cu-Gly100, Cu-Gly75, and Cu-Gly50 groups. A decrease in the trabecular number in the Cu-Gly75, Cu-Gly50, and Cu-Gly25 groups was observed, compared to the Cu-Gly100 and control groups (Table 4).

Furthermore, a decrease in the real bone volume in metaphysis linked with a decrease in the mean trabecular thickness was observed in the Cu-Gly100 group, compared to the control group (S-Cu). However, an increase in the maximal trabecular thickness in the Cu-Gly50 and Cu-Gly25 groups was noted, compared to the Cu-Gly100 group. Additionally, the highest increase in the trabecular space was observed in the Cu-Gly50 group, compared to other groups (Table 4). Moreover, a decrease in the trabecular space in metaphysis was found in the Cu-Gly75% and Cu-Gly25 groups, compared to the other groups (Table 2). The trabecular number did not change after the Cu-Gly-treatment, irrespective of the concentration (Table 4).

Morphology of articular and growth plate cartilages

The analyzed joints had no visible lesions or degenerative changes. The Cu-Gly-treatment of adolescent rats (regardless of its dose) significantly decreased the total measured zones of the articular cartilage, compared to the control group supplemented with S-Cu (Figure 3). On the other hand, the influence of Cu-Gly on the total thickness of the growth plate was concentration dependent, and an increase was noted in the Cu-Gly50 group compared to the Cu-Gly75 group, and in the Cu-

Gly25 group compared to the control and Cu-Gly75 groups. In turn, the thickness of zone I decreased in the Cu-Gly100 group, compared to the control group and increased compared to other Cu-Gly-treated groups (Figure 3). Moreover, a decrease in the thickness of zone II was noted in the Cu-Gly75 group, compared to the other groups, and elongation of this zone was reported in the Cu-Gly50 group, compared to the Cu-Gly100 group (Figure 3). Zone III was higher in Cu-Gly50 compared to the Cu-Gly75 group and the S-Cu supplemented group. Zone IV was shorter in the Cu-Gly75 and Cu-Gly50 groups, compared to the control group (S-Cu), and longer in the Cu-Gly25 group (Figure 3).

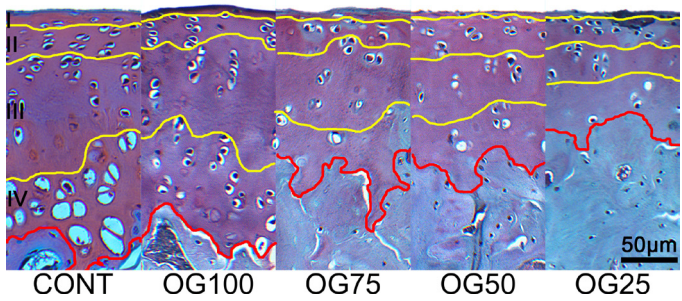


Description of the groups as in Figure 1.

Figure 3. Morphology of articular cartilage and growth plate of femur obtained from 18-week-old rats treated with different levels of Cu-Gly

Proteoglycan content in articular cartilages

Proteoglycan staining with SO revealed lower content of proteoglycans (weaker staining) in the cartilage from the Cu-Gly50, Cu-Gly25, and Cu-Gly75 groups. In turn, rats from the Cu-Gly100 demonstrated moderate to very strong staining associated with the higher content of proteoglycans. The concentration of proteoglycans in the Cu-Gly supplemented groups, irrespective of its dose, exhibited a gradual increase along the distance from the periphery of the cartilage and loss of SO staining. In the control rats supplemented with S-Cu, there was no evident gradient in safranin O staining, and their articular cartilages had strong intensive red staining, compared to the other Cu-Gly supplemented groups (Figure 4).



I – horizontal (superficial surface); II – transitional; III – radial; IV – calcified zone.
The cartilage from the Cu-Gly50, Cu-Gly25, and Cu-Gly75 groups displayed lower proteoglycan content (displaying weaker staining), while the Cu-Gly100 rats demonstrated moderate to very strong staining linked with higher content of proteoglycans. The concentration of proteoglycans in the Cu-Gly supplemented groups, irrespective of its dose, exhibited a gradual increase with the distance from the periphery of the cartilage and loss of SO staining. In the control rats supplemented with S-Cu, there was no evident gradient in safranin O staining, and their articular cartilages exhibited strong intensive red staining, compared to the other Cu-Gly supplemented groups.
Description of the groups as in Figure 1.
Red lines indicate the bottom border of articular cartilage whereas white arrow indicated its thickness.

Figure 4. Representative images of safranin-O staining carried out on formaldehyde-fixed sections from femoral articular cartilage of 18-week-old rats treated with different levels of Cu-Gly

According to the Mankin semi-quantitative score system, the cartilage of the rats from the Cu-Gly25, Cu-Gly50, and Cu-Gly75 groups had surface irregularity and loss of safranin O staining, however without structural changes, which gave a score of 3, compared to the control and Cu-Gly100 groups (Figure 4).

Discussion

Dietary studies concerning the amino acid complex are required, because traditional inorganic mineral salts including sulfates, oxides, and carbonates are used at 10-fold higher doses than the recommended ones in order to avoid trace mineral deficiency (Tomaszewska et al., 2016 c). On the other hand, some studies report greater bioavailability of organic sources, thus it should allow decreasing their supplemented amount in the diet (Świątkiewicz et al., 2001; Andersen, 2004). A study conducted by Kwiecień et al. (2014) shows that the use of a considerably reduced level of Cu in the form of glycinate compounds instead of sulfate at the recommended dose does not influence the physical, chemical, mechanical, and morphometric properties in chicken bones. Nevertheless, further investigations will be required to assess how efficiently the skeletal system will adapt to reduced Cu intakes. There are many effects associated with acquired Cu deficiency. Currently, the prolonged use of zinc supplements, which may cause secondary copper deficiency, requires special

considerations (de Romaña et al., 2011). Moreover, low Cu status has been associated with bone malformation during development and risk of developing osteoporosis in later life (de Romaña et al., 2011). There are reports of abnormal growth or bone development in Cu-deficient animals, but it appears that children and some species such as chickens, dogs, horses, and rabbits are more sensitive than rats (Beattie and Avenell, 1992).

It is known that a proper Cu status in the living organism is necessary to reach genetically optimal growth. Moreover, at present, there is a clear need to improve the current knowledge about early effects of both deficient Cu intake and excess Cu exposure. There is a variety of indicators for recommended levels for Cu e.g. the plasma Cu concentration, serum ceruloplasmin, superoxide dismutase activity, or platelet Cu concentration (Palacios, 2006). However, blood plasma or ceruloplasmin concentrations alone are relatively insensitive markers. To analyze the metabolism of copper fully, the concentration of Cu in blood serum, urine, and liver as well as the concentration of ceruloplasmin should be known (Baker et al., 1999; Armstrong et al., 2004). Instead, it seems that the Cu liver content is a better indicator of the Cu status. Our earlier study shows that the Cu content in the liver is not affected by Cu-deficient diet (in the Cu-Gly form) in adolescent rats (Tomaszewska et al., 2014).

The adolescent rats in this study exhibited unaffected plasma Cu concentrations after the Cu-Gly supplementation, irrespective of the dose used. Additionally, the Ca, P, and Zn concentrations were not affected, but the Fe concentration was decreased after 12 weeks, irrespective of the dose used. There is a Cu-Fe link, which is well documented in nutritional studies. One such example is the multi-copper ferroxidase, hephaestin, which plays an important role in intestinal iron transport. Hephaestin activity is decreased in the intestine of Cu-deficient mice, which correlates with systemic iron deficiency (Chen et al., 2006). Other studies have also shown that copper deficiency reduces iron absorption in rats (Reevers et al., 2004). Probably, there may have been a problem with intestinal Fe absorption in our rats. Moreover, our results are in agreement with another study, where Zn absorption and retention were generally not affected by the addition or sources of Cu (Apgar and Kornegay, 1996). After the 12-week Cu-Gly-treatment, the adolescent rats fed with the diet supplemented with Cu-Gly at 75% of daily requirement were the heaviest. This finding is also in agreement with another study performed on adolescent rats supplemented with Cu as a chelate at the level of 75% of the required daily amount (Tomaszewska et al., 2014; Tomaszewska et al., 2016 b). Additionally, this is consistent with a recent study indicating that an organic form of Cu (Cu citrate) stimulates growth at lower concentrations than that of S-Cu in broilers and swine (Pesti and Bakalli, 1996; Armstrong et al., 2004). On the other hand, other studies report that Cu deficiency in relation to daily requirement leads to reduced body mass because Cu is essential for normal growth and development (Uauy et al., 1998).

Furthermore, the bone Cu content was found to correlate negatively with bone Ca concentration or bone density in ageing mice (Massie et al., 1990), while the Cu level in human osteoporotic bone was found to be unchanged or slightly higher than in normal bone in subjects at the same age (Beattie and Avenell, 1992). In our study, there were no differences in the Ca, P, and Cu bone content after 12 weeks of

the Cu-Gly-treatment in adolescent rats. A comparison of these findings with those from other studies is somewhat complicated because no studies of the effects of the dietary Cu-Gly form at different concentrations below daily requirement have been reported. Our study has shown impaired bone development of all Cu-Gly-treated adolescent rats. However, the femora of rats from the Cu-Gly75 and Cu-Gly50 groups were shorter and lighter than in the other animals. Different results were obtained in a study with Cu-deficient chickens showing no difference not only in body weight but also in the basal morphology of tibia between the Cu-deficient and control chickens (Riggins et al., 1979). There is also another study performed with Cu-Gly given to growing chicken for 6 weeks at different levels of total requirement (100%, 50%, and 25%), which shows that the reduction of dietary Cu-Gly to 25% results in an increase in bone weight but without changes in the length (Kwiecień et al., 2014).

In our study, significant alteration was noted in bone morphology and geometry, which was Cu-Gly-dose dependent. Our rats supplemented with dietary Cu-Gly had less immature bone with a thicker wall (regardless of the concentration), which caused a decrease in bone mechanical endurance. There was a 15% and 21% decrease in the ultimate and maximal elastic strength in the Cu-Gly100 and Cu-Gly75 groups, respectively, compared to the S-Cu group. This was the strongest effect noted among the Cu-Gly supplemented groups. However, the 50% Cu-Gly administration enhanced maximal elastic strength by 11%, compared to the S-Cu group. In general, it can be proved that the supplementation of Cu-Gly, even in a deficient amount, compared to the recommended dose, negatively influences the process of bone development and results in worse mechanical endurance in adolescent rats. Our adolescent rats treated with Cu-Gly at a Cu-deficient level exhibited strongly osteoporotic cancellous bone in a dose-dependent manner.

This is in contrast with the effect described in adult rats, where Cu-deficient diet enhanced mechanical endurance and simultaneously triggered an osteoporotic effect in cancellous bone (Tomaszewska et al., 2015). It was also shown that although the ash weight and the calcium content of femora from the Cu-deficient animals were similar to those in the controls, mechanical endurance was reduced (Jonas et al., 1993). Probably other mechanisms are involved in reduced mechanical endurance, because Cu is required for proper growth manifested by achievement of appropriate bone mass and strength (Uauy et al., 1998). Impaired mechanical strength is related to defects in collagen, whose cross-links are essential for bone to possess a sufficient deflection capacity, bending strength, and stiffness (Baxter and Van Wyk, 1953; Jonas et al., 1993; Oxlund et al., 1995). The bone mass in a part of the skeleton is directly dependent upon both its volume or size and the density of mineralized tissue. Additionally, the external diameter of bones, which enlarges with aging, influences mechanical properties. This phenomenon has been documented by measuring the external diameter of several bones (Bonjour et al., 1994).

Our study also presented the results of the growth plate measurement, which indicated that the Cu-Gly complex influenced the growth plate in a dose-dependent manner. The reduction of the proliferative zone indicated disturbances in the division and differentiation of cells caused by a decrease in chondrocyte metabolism. The reduction of zone IV may suggest a decreased degree of mineralization. As

a result, changed trabecular architecture and reduced cancellous bone were observed. The present study also showed a significant alteration in the articular cartilage. The worst effect was the shortening of the superficial zone, which can influence the distribution of the load through the joint and result in degradation of articular cartilage causing difficulties in movement. Furthermore, it can provoke irreversible deformation caused by the impact of the load during movement. It should also be mentioned that surface irregularity in articular cartilage was observed. Additionally, there was observed reduced proteoglycan content in the articular cartilage (Figure 5). The degradation of proteoglycans involves destabilization of the collagen network. Proteoglycans give stability to articular cartilage and their content in cartilage is directly proportional to the intensity of safranin staining. Thus, this method can be used to demonstrate any changes in articular disease (Camplejohn and Allard, 1988).

A good side of this experiment was the use of various chemical forms of Cu, i.e. the reference group was treated with Cu at the same dose but in the form of S-Cu, and different Cu sources have different relative bioavailability (Banks et al., 2004) and the mechanism and extent of growth-promoting effects are fairly varied. In these studies, copper sulfate (S-Cu) was used as a reference point for comparison of the relative bioavailability of various Cu sources (Banks et al., 2004).

To the best of our knowledge, this is the first study that has examined both the mechanical properties of bone and the histomorphometry of cancellous bone and hyaline cartilage in not only Cu-deficient diet but also diet containing varied sources of Cu given at similar levels in adolescent rats.

Conclusions

No studies conducted so far have provided a detailed analysis of the histomorphology of bone and hyaline cartilage in adolescent rats administered with diet containing different Cu forms and doses. Irrespective of the dose, the organic form can lead to changes within the articular cartilage, as indicated by morphological analysis and proteoglycan content. Moreover, the Cu-Gly treatment influenced the growth plate and trabecular architecture. It appears that the use of the dietary Cu-Gly complex at a substantially reduced dose instead of Cu added in the form of sulfate according to recommended dose did not inhibit the development of bone and hyaline cartilage in adolescent rats.

Conflict of interest

There are no known conflicts.

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Received: 25 III 2017
Accepted: 27 VII 2017