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Alterations of liver histomorphology in relation to copper supplementation in inorganic and organic form in growing rats

Ewa Tomaszewska[1], Piotr Dobrowolski[2], Małgorzata Kwiecień[3], Natalia Burmańczuk[1], Barbara Badzian[1], Sylwia Szymańczyk[1], Paulina Kurlak[2]

[1]Department of Animal Physiology, Faculty of Veterinary Medicine,
[3]Institute of Animal Nutrition and Bromatology,
University of Life Sciences in Lublin, 20-950 Lublin, Poland
[2]Department of Comparative Anatomy and Anthropology,
Maria Curie-Skłodowska University, Lublin, Poland;
ewaRST@interia.pl

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Abstract

The aim of this study was to define the effects of diet containing the same mineral content of mineral salt or amino acid chelate, and diet containing various levels of Cu amino acid chelate on liver histomorphometry in growing rats. Male Wistar rats were used in the 12th week experiment. The control group (n = 12) was fed standard diet, which provided Cu in an inorganic form at the level required for rats. The experimental animals were divided into four groups (each n = 12) depending on different levels (100%, 75%, 50%, 25% covered daily demand) of Cu supplementation in chelated form. Cu content was determined in the liver tissue and blood plasma. Immunohistochemical staining with caspase-3 antibody was performed. Microscopic assessment of the liver structure indicated that Cu supplementation did not change the liver architecture. However, histomorphometric analysis revealed a significant increase in the number of nuclei, total cell number, and multinucleated hepatocytes in rats supplemented with the organic form of Cu at the level of 25% compared with the control group. There was a considerable increase in the number of apoptotic cells and ballooning degeneration of hepatocytes, especially in groups supplemented with organic form of Cu covering the daily demand in 100% and 75%, in comparison to control group. Moreover, there was no Cu deposition in the liver and changes in Cu content in blood. Cu provided in the diet in organic form covering an amount of its minimum daily demand in 25% appears to be the least harmful with regard to the liver. It indicates that there is a need to establish the level of diet supplementation with Cu amino acid chelates.

Keywords: rats, liver, cooper, amino acid chelates, histomorphometry.

Introduction

Copper (Cu) is the third most abundant essential trace mineral in the body after iron and zinc. Cu is an essential micronutrient required by all living organisms and it is present in all animal tissues. The highest concentration of Cu is in the liver, kidneys, brain, and heart, and the lowest content is in bones and muscles. The average content of Cu in tissues of adult mammals is three times lower than in growing animals. This content and distribution is changed throughout the animals' life. Infancy is a very critical period in life due to increasing demand of Cu during rapid growth; whereas, diets can provide low amounts of this element, and it may lead to limitation that persists in adulthood (19, 24). The imbalance of Cu homeostasis in humans and animals causes different health problems, like neurodegenerative symptoms, alterations in cholesterol metabolism, and cardiovascular structural and functional problems (2, 6). The deregulation of Cu metabolism alters responses in the processes of inflammation, infection, or cancer. Cu is an essential cofactor in a number of enzymes, *i.e.* superoxidase dismutase (SOD), cytochrome oxidase (COX), and ceruloplasmin (CP). Its role in connective tissue synthesis is linked to the enzyme - lysyl oxidase (3, 10, 21, 22, 26). Moreover, Cu plays a direct role in carbohydrate metabolism. Cu deficiency can lower

hepatic glycogen and increase blood glucose levels. Cu also plays an important role in iron (Fe) metabolism by competitive inhibition in its transport and bioavailability (3). On the other hand, Cu as an ion is toxic and generates free radicals (4).

Cu absorption occurs in all segments of the gastrointestinal tract. Typically, the gastrointestinal tract absorbs about 50% to 80% of ingested Cu. Studies on humans and animals show that this absorption is regulated by the nutritional status and it is influenced by the chemical form in which the element is present. Most dietary Cu passes through the liver where it can be used for protein and energy production, and is excreted by the biliary route or in a small amount in the urine. Liver Cu concentrations can be elevated to concentrations that cause toxicosis in stressful conditions, and affect liver functions. Copper accumulation in the liver takes place in the Wilson's disease, which causes liver damage and degeneration (11, 14, 25). On the other hand, oxidative stress as a consequence of Cu deficiency has been linked with a faster decline in cognitive ability in Alzheimer's disease (13).

Several different Cu sources can be used in the trace mineral premix for animals. They can be divided into inorganic sources, such as copper sulfate or carbonate, and organic sources, such as a chelated form with higher Cu bioavailability (7-9, 15, 18, 20). Absorption of Cu is enhanced by amino acids and is more effective than minerals that are present in the cationic state. It seems to be due to the fact that cationic minerals must be chelated by proteins in the cell wall prior to absorption, thus slowing down the process. No additional chelation of the amino acid chelates is required at the brush border of the cell membrane, thus making the membrane transport more rapid. Moreover, digestion can lead to the formation of insoluble compounds. Phytates and fiber from grain-based foods can reduce mineral solubility and availability (30, 31). Furthermore, the combination of dietary molybdenum and sulfur along with iron reduces the absorption of dietary Cu. The consumption of a great amounts of zinc also can reduce the absorption of Cu, causing a deficiency of this element.

The aim of the study was to define the effects of diet containing the same Cu content in the form of mineral salt and amino acid chelate, as well as the effect of diet containing different levels of Cu amino acid chelate on histomorphometrical parameters of the liver in growing rats.

Material and Methods

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

Animals, breeding and experimental design. Sixty male Wistar rats weighing 217.9 ± 25.5 g at the age of five weeks were used in the study. The experiment lasted 12 weeks (excluding an acclimatisation period in the first week). Clinically healthy rats were individually kept in Macrolon cages at 21 ± 1°C and 55% humidity, and 12-h light and dark cycles. The rats were randomly divided into control and experimental groups. Control group (n = 12) was fed standard diet (LSM, AGROPOL S.J., Poland), which provided Cu in inorganic form in levels required for rats (5 mg/kg b.w. per day). Experimental animals were fed the same standard diet but without Cu. Basal composition of diet administered during the study was as follows: min. 14.5% of crude protein, min. 1.5% of crude fat, 10% of ash, and min. 5% of crude fibre. The composition of vitamin and mineral premixes of the diet is presented in Table 1.

Table 1. The composition of vitamin and mineral premixes of the diet (per kilogram of dry matter) administered to experimental rats during the study

Components	Per 1 kg of premix
Manganese, mg	5000
Iron, mg	5000
Zinc, mg	2500
Iodine, mg	75
Pantothenic acid (D-calcium pantothenate), mg	900
Retinol acetate (UI)	800 000
Cholecalcipherol (UI)	100 000
Tocopherol, mg	4964
Menadione sodium bisulphite, mg	300
Riboflavin, mg	600
Pyridoxine HCL, mg	60
Cyanocobalamin, mg	1.2

Supplementation of Cu amino acid chelate. Experimental animals were divided into four groups (each n = 12) depending on the level of Cu supplementation (Table 2). Copper was given to water as Cu amino acid chelate (Cu-glycine complex). All animals had a free access to water.

The level of Cu required for rats: 5 mg/kg b.w. per day in water, was calculated according to earlier studies (17, 21, 22). Water consumption during 24 h was measured before the beginning of the experiment, and the data was used to calculate the administered amount of Cu. This data, combined with body weight, Cu content in the chelate, and the amount of the chelate

covering Cu demand, was used to calculate and maintain the amount of Cu at the dose of 5 mg/kg b.w./day in tap water. There were no differences in water consumption between the groups. At the end of experiment, rats were fasted for 24 h and euthanised individually with carbon dioxide inhalation and by dislocation of the spine.

Table 2. The arrangement of the study

Group	Treatment
I	control group – 100% of daily demand of Cu in inorganic form
II	0.025 mg Cu*/L H_2O - 100% of daily demand
III	0.01875 mg Cu*/L H_2O - 75% of daily demand
IV	0.0125 mg Cu*/L H_2O - 50% of daily demand
V	0.00625 mg Cu*/L H_2O - 25% of daily demand

* as Cu-Gli in distilled water; the chelated copper covering 100% of daily demand and inorganic salts contained an equivalent mineral concentration

Blood plasma analysis, liver tissue collection, and histomorphometric analysis. Blood samples were drawn by the heart puncture. Obtained plasma samples were stored at -25°C until further analysis. The plasma concentration of Cu was determined by a colorimetric method using a Metrolab 2300 GL unit (Metrolab SA, Argentina) and sets produced by BioMaxima (Poland).

Liver samples (0.5 cm^3) were prepared for histological examination as previously described (32), except for the samples which were cut to 4 µm sections and stained with haematoxylin and eosin (HE), and Masson's trichrome method (MT). Microscopic (two-dimensional) images were collected using a microscope AXIOVERT 200M (Carl Zeiss, Germany), equipped with a camera AxioCam HRc (Carl Zeiss, Germany). The structure of the liver was examined under microscope and the images collected were analysed with the use of graphical analysis software CellSens Olympus Version 1.5 (OLYMPUS, Japan) and ImageJ 1.48r (National Institute of Health. USA, http://rsb.info.nih.gov/ij/index.html). The following parameters were analysed: intercellular space as an area (%); fractal dimension of intercellular space (D); total number of cells/mm^2; total hepatocyte number/mm^2; not-hepatocyte cell number/mm^2; hepatocyte nuclei number/mm^2; number of mononuclear hepatocytes/mm^2; and number of multinucleated hepatocytes/mm^2.

Determination of Cu content. Liver sample weighing about 5.0 g ±0.001 g was put into quartz crucibles and burnt at 450°C. Then, the resulting ash was dissolved in a specified volume of 1 M of nitric acid. The Cu content was determined by means of an Avanta PM flame atomic absorption spectrophotometer (GBC).

Immunohistochemistry. The sections selected for immunohistochemistry were deparaffinised, rehydrated, and microwaved 3×5 min in 10 mM citrate buffer, pH 6.0, to retrieve antigenicity. Non-specific binding was blocked with 5% goat serum (Sigma) for 1 h at room temperature. Afterwards, overnight incubation at 4°C with rabbit polyclonal antibodies against the active form of caspase-3 (Abcam, UK, dilution 1:200) was used as the primary antibody to indicate apoptotic cells. Biotinylated anti-rat immunnoglobulin (DacoCytomation, Denmark, dilution 1:200) was used as the secondary antibody. The control sections were incubated in the absence of the primary antibody. Such omission resulted in no deposition of the reaction product. Apoptotic cells were counted per square millimetre of tissue.

Statistical analysis. All results were expressed as means ± SD (standard deviation). Differences between means were tested with One Way ANOVA, and post hoc Dunnett's test was used 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. $P < 0.05$ was considered statistically significant. All statistical analyses were conducted by means of STATISTICA 8.0 software (StatSoft, Inc. (2008). STATISTICA (data analysis software system), version 8.0. www.statsoft.com).

Results

Food consumption was measured daily in control animals and rats supplemented with Cu. No difference was observed between the examined groups.

Body and liver mass. The final body mass of the control rats and animals belonging to groups II, III, IV, and V was 391.5 ± 44.8 g, 416.6 ± 49.0 g, 429.0 ± 60.8 g, 421.2 ± 52.4 g, and 446.0 ± 74.5 g respectively. There was no effect of Cu supplementation on liver mass. However, at the end of the study, rats from control group receiving 100% of Cu daily demand administered in inorganic form appeared smaller in size, but no signs of morbidity were observed.

Cu plasma and liver content. The Cu plasma concentrations of the control rats and animals belonging to groups II, III, IV, and V were 27.65 ± 3.22 µmol/L, 29.04 ± 3.73 µmol/L, 29.87 ± 6.18 µmol/L, 29.34 ± 4.42 µmol/L, and 30.9 ± 6.66 µmol/L, respectively. The Cu liver contents of the control rats and animals belonging to groups II, III, IV, and were 0.24 ± 0.004 µg/g, 0.26 ± 0.008 µg/g, 0.27 ± 0.006 µg/g, 0.26 ± 0.007 µg/g, and 0.27 ± 0.008 µg/g respectively. There were not differences in Cu plasma and liver content between the control group and Cu deficient animals.

Histomorphometry of the liver and immunohistochemical analysis. Histomorphometrical parameters of the liver are presented in Table 3. Microscopic assessment of liver structure showed no marked differences in distribution of portal triads and terminal hepatic venules in comparison to controls. Moreover, Cu supplementation did not change the general lobular architecture under low microscopic magnification. However, irregularity in hepatocyte cords was observed in groups II, III, IV, and V when compared with control group, as well as hepatocyte vacuolisation was noted in groups II, III, and IV (Fig. 1). Histomorphometric analysis also showed an increase in collagen amount in the group V supplemented with Cu amino acid chelate in 25% of daily demand (Table 3). More detailed examination revealed a large increase in the number of apoptotic cells, especially in group II (17 fold increase) and group III (4 fold increase) in comparison to control group. In contrast, group V with the lowest amount of Cu in chelated form, had the lowest number of degenerated hepatocytes among experimental groups. Moreover, an increase in ballooning degeneration (Fig. 2) especially in group II (twofold increase) and group III (threefold increase) was observed, as well as 39% increase in group IV, comparing to the control group. In contrast, group V, with the lowest amount of Cu in chelated form, had by 69% less degenerated hepatocytes than control group. In addition, ballooning and vacuolisation were mostly observed in the third and rarely in the second zone regarding Rappaport`s liver acinus**,** which corresponds to a progressive decrease in tissue oxygenation. Furthermore, in groups II, III, IV, and V, the number of small hepatocytes increased by 50%, 100%, 44%, and 20% respectively, in comparison to control group. Histomorphometric analysis revealed a significant increase in the number of nuclei, total cells, and multinucleated hepatocytes in rats supplemented with organic form of Cu at the level of 25% of daily demand compared with control group supplemented with inorganic form (Table 3).

Table 3. Histomorphometrical parameters of liver tissue of rats from control group and groups treated with different levels of Cu daily demand, administered in chelated form

Parameter	Group I	Group II	Group III	Group IV	Group V
Number of nuclei/mm^2	1611 ± 231	1739 ± 309	1732 ± 243	1730 ± 245	1835 ± 155*
Total number of cells/mm^2	2254 ± 306	2351 ± 305	2196 ± 243	2360 ± 269	2482 ± 162*
Total number of hepatocytes/mm^2	1581 ± 230	1691 ± 292	1674 ± 318	1681 ± 230	1766 ± 150
Number of mononuclear hepatocytes/mm^2	1551 ± 230	1643 ± 276	1617 ± 195	1631 ± 218	1697 ± 150
Number of multinucleated hepatocytes/mm^2	19.25 ±3.51	27.21 ±4.66	28.97 ±5.79*	28.59 ±5.72	25.78 ±5.76*
Number of non-hepatocyte cells/mm^2	673 ± 139	659 ± 108	521 ± 148	678 ± 113	716 ± 67
Intercellular space as an area (%)	6.96 ± 3.43	10.98 ±4.58*	7.95 ± 3.25	6.58 ± 3.03	5.69 ± 1.54
Fractal dimension of intercellular space (D)	1.43 ± 0.12	1.52 ± 0.09*	1.46 ± 0.15	1.42 ± 1.12	1.37 ± 0.06
The area of collagen μm^2/mm^2 of tissue	5395 ± 3622	4297 ± 1763	4150 ± 4234	5803 ± 2949	9010 ± 4882*
Apoptotic cells/mm^2	23 ± 9	382 ± 128*	107 ± 27*	64 ± 25	44 ± 23

Presented data are means ±SD, * $P < 0.05$, groups II, III, IV, and V *vs.* control group I

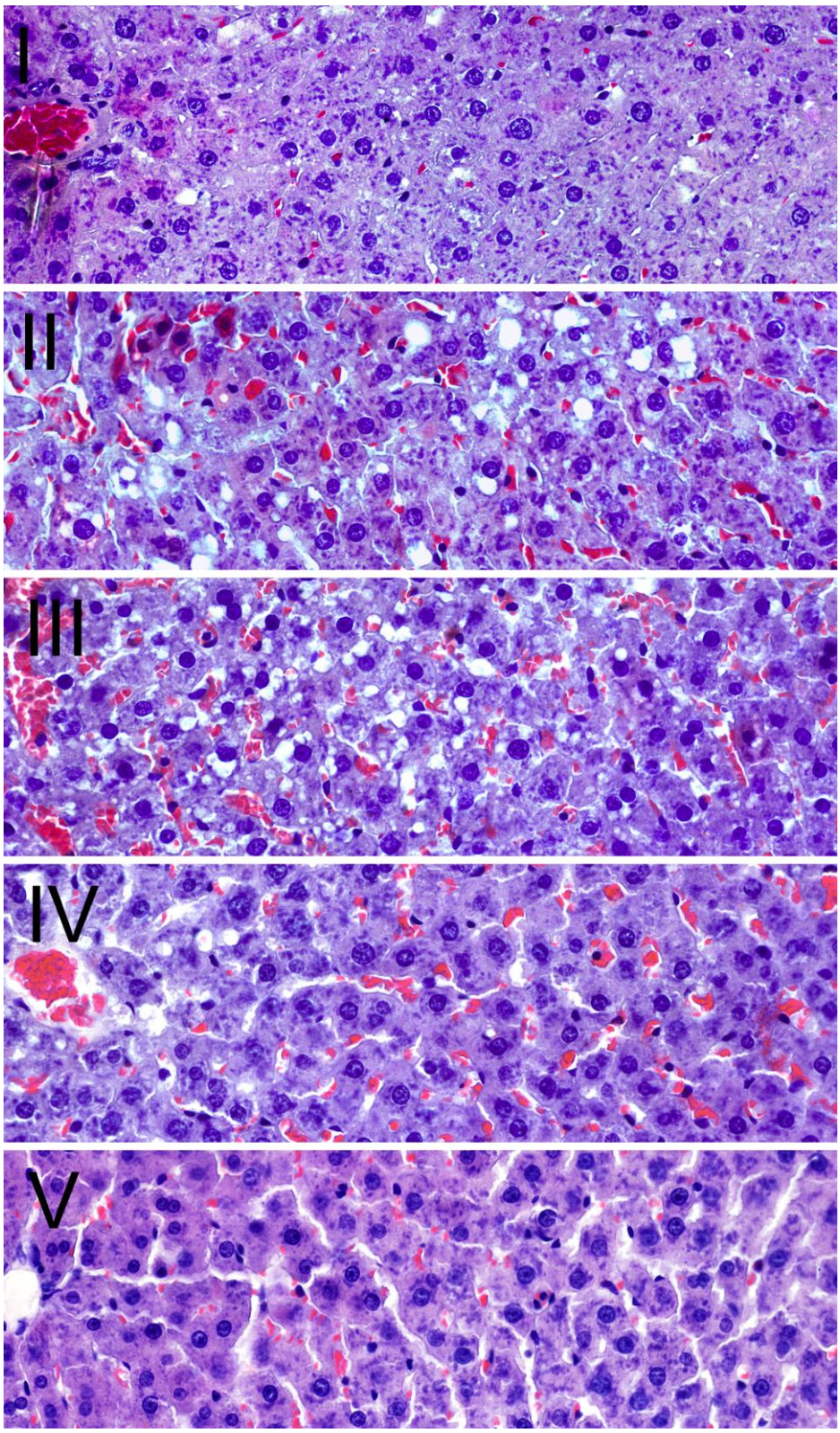


Fig. 1. Sections of the liver of rats supplemented with Cu in inorganic form in 100% of daily demand (I – the control group) and Cu amino acid chelate in 100% of daily demand (II); 75% of daily demand (III); 50% of daily demand (IV), and 25% of daily demand (V). H.E., 200×

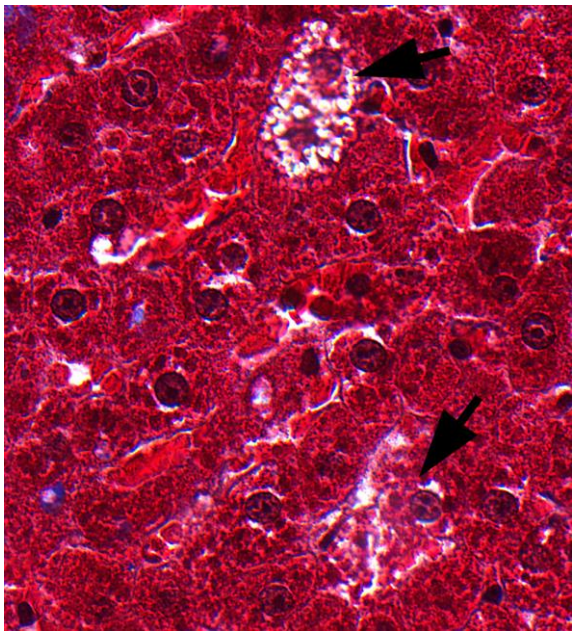


Fig. 2. Ballooning degeneration of hepatocytes. Arrows indicate degenerating hepatocytes. M.T. staining, 400×

Discussion

The access to copper from the environment is limited in humans and many animal species. Cu content in the diet varies widely because foodstuffs differ greatly in their natural content of this element. Food and drinking water and Cu-containing supplements are the main sources of Cu. Its deficiency in infants is associated with low haemoglobin content. Low Cu status has been also associated with bone malformation during development, risk of developing osteoporosis in later life, impaired melanin synthesis, poor immune response, susceptibility to infections, poor cardiovascular health, alterations in cholesterol metabolism. Clinically, Cu deficiency has been described in infants on total parenteral nutrition without adequate mineral supplementation, and in malabsorption syndrome or persistent nephrotic syndrome that increases copper loss (13). Cu deficiency occurs early in life when high requirement for rapid growth is observed, and the liver stores are reduced, but the supplementation of groups at the risk for Cu deficiency is discussed. Thus, it is necessary to examine the effects of supplementation of this metal amino acid chelate on liver histomorphometrical changes under conditions of different levels of deficient Cu intake.

In the study, one of the diets had very low Cu content, thus it was considered to be deficient in its amount. Cu in chelated form was added to feed to provide experimental diets with different levels of the needed amount (25%, 50%, 75%), which completely covered its daily demand. The present results did not show any direct effect of dietary Cu content on the liver mass. Cu supplementation at experimentally deficient dose, administered in chelated form also did not affect the body weight.

These results were contrary to those of Megahed *et al.* (17) who reported a decrease in the body weight in rats fed Cu deficient diet as compared to diet covering the daily demand. This decrease in body weight indicates that Cu is essential for normal growth and development as an integral part of many enzymes, which are dependent on Cu for their normal activity. However, another study revealed that a diet marginally deficient in Cu did not affect the feed intake and weight gain of rats (16).

Cu homeostasis is maintained by the support of several organs like the intestine, liver, and kidneys. The central organ of Cu metabolism and storage is the liver, the most sensitive organ for Cu deficiency (11, 14). The distribution of Cu in organs and tissues was investigated in numerous studies (27). However, it is difficult or impossible to assess marginal copper status in humans and animals as well (5). Rats are often used as models for dietary Cu deficiency in humans, because Cu deficiency or toxicity is rare in humans, the extent of marginal deviations from optimum copper status, and any consequent clinical health effects are uncertain. Therefore, it is necessary to conduct histo-morphometrical analysis in conditions of various Cu deficiencies .

In the present study, although dietary Cu amino acid chelate supplementation in deficient amount did not influence the growth of animals or development of the liver, it affected the liver structure. Any changes in size and shape of hepatocytes could be considered as a sign of changed metabolic activity. Moreover, changes were described in the acini in zones 2 and 3 corresponding to progressive decrease in tissue oxygenation. Thus, apoptosis and swollen hepatocytes were observed. This cell swelling is not genetically regulated as a passive cell death, relating not only to single cells but also to large groups of cells, even entire organs. Regeneration of tissue or fibrosis could be the final reaction. Restriction of dietary Cu in rats may induce hepatic steatosis, although it was not observed in this study. Moreover, liver cells showed multiple nuclei that also could be considered as a sign of increased metabolic activity.

Glycogen storage disease, also known as glycogenosis, is characterised by deficient or defective activity of the enzymes responsible for metabolising glycogen in the body. It is a rare inherited disorder, which leads to accumulation of glycogen in the body. This abnormal accumulation of glycogen in tissues can result in the enlargement and dysfunction of various organs, including the liver, heart, and kidneys.

On the other hand, the chemical properties that make Cu biologically useful are also potentially toxic, especially when Cu taken with food excesses the amounts necessary for life (12, 28). Therefore, there is a need for effective, well-tolerated alternative chelating

agents to control the Cu accumulation, which occurs, for example, in some chronic liver diseases (6). Impaired hepatic excretion of Cu leads to its accumulation in the liver, which is observed in Wilson`s disease with a decrease in serum concentration of the element. It can also lead to neurological problems, hepatitis, cirrhosis, and finally to death. It is an inherited disorder of Cu metabolism with two mutated genes (23).

In animals used in the present experiment, no Cu deposition in the liver was noted, because there was no difference in Cu content in the liver and plasma between control group supplemented with inorganic form of Cu in 100% of daily demand and groups supplemented with organic form of Cu at various levels of deficiency, particularly in a group group administered with chelated Cu in 100% of daily demand. It may indicate that there was no excessive Cu absorption and storage in investigated animals.

A major source of Cu in animal and human diets is supplemental Cu added to diets or free choice mineral supplements including cupric sulphate, cupric carbonate, cupric oxide, and various organic Cu sources. Amino acid chelates are reported to have significantly higher absorption rates from the intestines compared to soluble inorganic metal salts, but supplementation of the diet is often difficult and economically unprofitable. Presumably, the use of chelated minerals with higher bioavailability can allow lower supplementation and reduced waste from unassimilated minerals (1).

It is apparent that there is a need to use more criteria to establish the level of Cu diet supplementation. Unfortunately, acute Cu deficiency is relatively rare in humans and animals fed typical diets. Chronic deficiency is much more common. Identification of the sign of subclinical Cu deficiency is the challenge. The determination of Cu requirements and insignificant deficiency is complicated by the fact that Cu deficiency cannot be linked with low level of Cu-dependent enzymes. The effect of subclinical Cu deficiency is not well defined, although it can play a role in numerous, common degenerative diseases and conditions. Moreover, long-term feeding studies assessing the influence of Cu amino acid chelates deficiency on liver histomorphometry are limited.

Cu given in the diet in organic form covering in 25% the minimum daily demand appears to be the least harmful with regard to the liver.

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