



THE EFFECT OF A RAT DIET WITHOUT ADDED CU ON REDOX STATUS IN TISSUES AND EPIGENETIC CHANGES IN THE BRAIN*

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Abstract

The aim of the study was to determine whether feeding rats a diet without added Cu increases oxidation of macromolecules in tissues, as well as epigenetic changes in the brain. The rats were divided into two groups: the Cu-6.5 group which was fed a diet with a standard content of Cu in mineral mixture – 6.5 mg Cu from CuCO₃ per kg of diet; and the Cu-0 group which was fed a diet with a mineral mix without Cu supplementation. At the end of the experiment the rats were weighed and blood samples were collected. Finally, the rats were euthanized and then the liver, small intestine, spleen, kidneys, heart, brain, lung, testes and leg muscles were removed and weighed. In the blood of Cu-0 rats the lower Cp activity and greater GPx and CAT activity than in Cu-6.5 rats were noticed. In the liver, lungs, heart and testes of Cu-0 rats, a decreased content of Cu were noticed. Application of Cu-0 diets resulted in increased LOOH level in the small intestine, liver, and heart, as well as increased MDA content in the liver, spleen, lungs, brain and testes. The Cu-0 treatment caused a decrease in SOD activity in the heart, lungs and testes of the rats and a decrease in CAT activity in the small intestine. In the brain and testes of rats from the Cu-0 treatment, lower content of GSH + GSSG was observed. The brain of rats from the Cu-0 treatment showed an increase in the level of PCs, 8-OHdG, Casp 8 and DNA methylation. The research has shown that a deficiency of Cu in the diet impairs the body's antioxidant defences, which in turn leads to increased lipid oxidation in the liver, small intestinal wall, heart, spleen, lungs, brain and testes, as well as to oxidation of proteins and DNA in the brain. A deficiency of Cu in the diet also increases methylation of cytosine in the brain.

Key words: copper deficiency, rats, brain, epigenetic change, redox status

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Copper is an element present in almost all cells of living organisms. It is found in the greatest amounts in the liver, brain and heart (Angelova et al., 2011; Gaetke et al., 2014). This element takes part in cellular respiration and is responsible for normal Fe metabolism and haemoglobin synthesis (Kumar et al., 2015, 2016; Ognik et al., 2018, 2019). Copper influences the immune system, in which it is involved in prostaglandin synthesis resulting from conversion of arachidonic acid and activation of neutrophils (Cholewińska et al., 2018 a, b; DiNicolantonio et al., 2018). In addition, copper is a cofactor of many enzymes which are crucial for the respiratory electron transport chain (cytochrome c oxidase), melanin, collagen and elastin synthesis (lysyl oxidase), iron metabolism (ferroxidase I and ferroxidase II) and antioxidant defense (ceruloplasmin, SOD1 and SOD3) (Angelova et al., 2011; Bost et al., 2016; Tishchenko et al., 2016). Cu also promotes proper functioning of the nervous system, in which it plays an important role in nerve myelination, synthesis of norepinephrine (copper dependent dopamine- β -hydroxylase) and endorphin activity (Brewer, 2010; Opazo et al., 2014; Kumar et al., 2015, 2016). In cultured rat olfactory bulb neurons and in rat cortical neurons copper modulates neurotransmission of different CNS neurons by blocking GABAergic and AMPAergic neurotransmission (Opazo et al., 2014).

The available literature indicates that very small amounts of Cu are needed to ensure normal functioning of the body. According to Food and Nutrition Board (FNB), the dietary daily copper intake for an adult should be at the level of 1.5–3.0 mg (NRC, 1989). It is estimated that the entire human body contains about 100 mg of this element (Bost et al., 2016). Both a surplus and a deficiency of copper can be extremely harmful to the body, causing a number of functional disorders (Gaetke et al., 2014; Bost et al., 2016; Cholewińska et al., 2018 a, b, c; Kodama et al., 2012). Uptake and distribution of copper is controlled by CTR1 and ATP7A or ATP7B transmembrane proteins. Even when copper is present in the diet the mutations in ATP7A gene block the delivery of copper to the secreted copper enzymes and an excretion of surplus copper from the cells (Tümer and Möller, 2010). In severely affected Menkes disease (MD) patients suffer from the lack of copper in vital organs such as heart, liver and brain, and reduced activity of essential cuproenzymes leads to death usually before the third year of life (Menkes et al., 1962). One of the symptoms of MD, the degradation of central nervous system and neuronal demyelination, is observed. There might be a relationship between this symptom and the role of copper in the formation of cytochrome c oxidase, SOD and lysyl oxidase because the brain ATP7A is involved in normal functioning of copper-dependent enzymes (Tümer and Möller, 2010). Moreover ATP7A and/or copper plays a role in axon outgrowth and synaptogenesis (El Meskini et al., 2007). In MD patients, copper is likely trapped in both the blood-brain barrier and the blood-cerebrospinal fluid barrier, while the neurons and glial cells are deprived of copper (Nishihara et al., 1998; Tümer and Möller, 2010). In addition, anaemia, leukopenia and damage to the skeletal and cardiovascular systems are observed in copper deficiencies (Aoki, 2004; Bost et al., 2016). A reduction in antioxidant enzyme activity due to copper deficiency may also impair antioxidant defence, leading to an increase in free radical reactions (Ognik et al., 2019). Copper deficiency in the prenatal period may result in impaired brain development. Characteristic neurodegenerative changes observed in developing rats with Cu deficiency

include impaired brain development, especially in the cerebellum, characterized by decreased myelination and synaptogenesis, as well as delayed development of the hippocampus and impaired motor functions (Gybina et al., 2009). Copper deficiency can also lead to isolated peripheral neuropathy, cerebral demyelination or optic neuropathy (Jaiser and Winston, 2010). Moreover, although the etiopathogenesis of Alzheimer's disease has not yet been fully explained, there is a clear correlation between its occurrence and low levels of Cu in the body. Biological material collected from patients with Alzheimer's disease shows significantly lower levels of Cu than in patients suffering from other neurodegenerative diseases, such as senile dementia (Klevay, 2008). It is likely that in conditions of copper deficiency, the multi-domain single-pass transmembrane protein APP present in brain cells, which is responsible for normal copper homeostasis, undergoes amyloidogenic processing and co-enrichment with amyloid-beta peptide. This leads to deposition of amyloid-beta and hyperphosphorylation of tau protein in the brain, resulting in the development of Alzheimer's disease (Hordyewska et al., 2014; Gamez and Caballero, 2015).

Excess of Cu in the body, like in Wilson disease, can also cause numerous metabolic disorders and dysfunctions in the body (Huster, 2010). High Cu levels lead to increased Fenton-type redox reactions, resulting in oxidative damage to cells and even cell death (Bost et al., 2016). Furthermore, excess Cu may cause disturbances in the lipid profile, damage to the gastrointestinal mucosa, and impairment of renal function (Chen et al., 2006). In addition, a high level of Cu in the body, like a Cu deficiency, leads to neurodegenerative changes in the brain (Scheiber et al., 2014). High content of copper is found in children on the autism spectrum (Bjorklund, 2013). Excess Cu can also lower the level of dopamine, which controls the pleasure and reward centres in the brain, while increasing the level of norepinephrine, which acts as a stress hormone. An imbalance of these two neurotransmitters may lead to autism symptoms as well as to anxiety, bipolar disorder, depression, ADHD, and paranoid schizophrenia (Walsh, 2012). Therefore, tight copper balance is indispensable.

Research on the effect of copper deficiency on the antioxidant defence in animals has already begun in the 1980s (Balevska et al., 1981; Lawrence and Jenkinson, 1987). A great deal of literature data indicates that both an excess and a deficiency of copper can lead to lipid oxidation in cells (Maiorino et al., 1995; Chauhan et al., 2008). Increased oxidation in the internal organs, e.g. the brain, liver and kidneys, leads to their dysfunction (Uttara et al., 2009; Lv et al., 2013; Cichoż-Lach and Michalak, 2014; Li et al., 2015; Muriel and Gordillo, 2016). Little is known, however, about whether copper deficiency also increases oxidation of proteins and DNA and whether it enhances epigenetic changes in the brain. Therefore, the aim of the study was to determine whether feeding rats a diet without added Cu increases oxidation of macromolecules in tissues, as well as epigenetic changes in the brain.

Material and methods

Animal protocol and dietary treatments

All animal care and experimental protocols were in compliance with current laws

governing animal experimentation in the Republic of Poland and with guidelines established by an ethics committee, in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, Directive 2010/63/EU for animal experiments, and were approved by the appropriate Local Institutional Animal Care and Use Committee.

Table 1. Composition of basal diet fed to rats (%)

Ingredient	Content
Invariable ingredients	
casein ¹	14.8
DL-methionine	0.2
cellulose ²	8.0
choline chloride	0.2
rapeseed oil	8.0
cholesterol	0.3
vitamin mix ³	1.0
maize starch ⁴	64.0
Variable ingredient	
mineral mix ⁵	3.5
Calculated content	
crude protein	13.5

¹Casein preparation: crude protein 89.7%, crude fat 0.3%, ash 2.0%, water 8.0%.

² α -Cellulose (SIGMA, Poznan, Poland), main source of dietary fibre.

³AIN-93G-VM, g/kg mix: 3.0 nicotinic acid, 1.6 Ca pantothenate, 0.7 pyridoxine-HCl, 0.6 thiamine-HCl, 0.6 riboflavin, 0.2 folic acid, 0.02 biotin, 2.5 vitamin B₁₂ (cyanocobalamin, 0.1% in mannitol), 15.0 vitamin E (all-rac- α -tocopheryl acetate, 500 IU/g), 0.8 vitamin A (all-trans-retinyl palmitate, 500,000 IU/g), 0.25 vitamin D₃ (cholecalciferol, 400,000 IU/g), 0.075 vitamin K₁ (phyloquinone), 974.655 powdered sucrose.

⁴Maize starch preparation: crude protein 0.6%, crude fat 0.9%, ash 0.2%, total dietary fibre 0%, water 8.8%.

⁵Mineral mixture with or without Cu, see Tables 2 and 3.

Table 2. Composition of mineral mixtures (MX) used in experimental diets (g/kg)

	MX with standard Cu dosage ¹	MX without Cu ²
Calcium carbonate, anhydrous CaCO ₃	357	357
Potassium phosphate monobasic K ₂ HPO ₄	196	196
Potassium citrate C ₆ H ₅ K ₃ O ₇	70.78	70.78
Sodium chloride NaCl	74	74
Potassium sulphate K ₂ SO ₄	46.6	46.6
Magnesium oxide MgO	24	24
Microelement mixture [#]	18	18
Starch	213.62	213.62
[#]Microelement mixture (g/100 g)		
Ferric citrate (16.7% Fe)	31	31
Zinc carbonate ZnCO ₃ (56% Zn)	4.5	4.5
Manganese carbonate MnCO ₃ (44.4% Mn)	23.4	23.4
Copper carbonate CuCO ₃ (55.5% Cu)	1.85	0
Potassium iodate KI	0.04	0.04
Citric acid C ₆ H ₈ O ₇	39.21	40.7

¹given to CuA group (5 weeks of feeding).

²given to CuD group (5 weeks of feeding).

Sixteen healthy male albino Wistar rats (Han IGS Rat [CrI:WI(Han)]), aged 5 weeks and with an average body weight of 135 ± 9.3 g, were randomly divided into two groups. The housing of animals and environmental conditions in animal laboratory were subject to current regulations (Fotschki et al., 2019). For 35 days the rats had free access to tap water and semi-purified diets, which were prepared and then stored at 4°C in hermetic containers until the end of the experiment (details in Table 1). The diets were modifications of a casein diet for laboratory rodents (AIN-93G) recommended by the American Institute of Nutrition (Reeves, 1997). Two experimental treatments were used to evaluate the effects of the presence or absence of added CuCO_3 in the diet.

The rats were divided into following groups: the Cu-6.5 group – the rats were fed a diet with a standard mineral mixture (MX) resulting in 6.5 mg Cu (from CuCO_3 in MX) per kg of diet; and the Cu-0 group – the rats were fed a diet with a mineral mix (MX) without Cu supplementation (CuCO_3 excluded from MX). The rats in each group (Cu-6.5 or Cu-0; 8 rats in each) received their respective diet for 35 days. The detailed composition of the mineral mixtures used in the experimental groups is given in Table 2.

All physiological measurements were made separately for each animal ($n = 8$ for each group). At the end of the experiment, the rats were fasted for 24 hours and anaesthetized *i.p.* with ketamine and xylazine (K, 100 mg/kg BW; X, 10 mg/kg BW) according to recommendations for anaesthesia and euthanasia of experimental animals. The rats were weighed and blood samples were collected from caudal vena cava. Finally, the rats were euthanized by cervical dislocation. Then the liver, small intestine, spleen, kidneys, heart, brain, lung, testes and leg muscles were removed and weighed. In the samples of collected tissues, the content of Cu were determined by inductively coupled plasma optical emission spectrometry (ICP-OES). The Certified Reference Material NIST-1577C Bovine liver was used for quality control. Moreover, homogenates were prepared from the small intestine, liver, brain, spleen, kidneys, heart, lungs, testes and leg muscles.

Redox status analysis in the blood

Markers of oxidative stress determined in the blood included lipid oxidation indicators, i.e. the concentration of lipid hydroperoxides (LOOH) and malondialdehyde (MDA), using adduct competitive ELISA kits produced by Blue Gene Biotech (Shanghai, China) and Cell Biolabs, Inc. (San Diego, USA), respectively. Activity of glutathione peroxidase (GPx) in the blood of the rats was determined spectrophotometrically using Ransel diagnostic kits manufactured by Randox (Poland). The method for GPx activity is based on a direct spectrophotometric assay where the peroxidase reaction is linked to glutathione reductase. The decrease in absorbance of NADPH at 340 nm is measured (Paglia and Valentine, 1967). A diagnostic kit manufactured by Oxis International, Inc., Portland, USA, was used to determine catalase activity (CAT). The sample containing catalase was incubated in the presence of a known concentration of H_2O_2 . After incubation for exactly one minute, the reaction was quenched with sodium azide. The amount of H_2O_2 remaining in the reaction mixture was then determined by the oxidative coupling reaction of 4-aminophenazone

(4-aminoantipyrine, AAP) and 3,5-dichloro-2-hydroxybenzenesulfonic acid (DHBS) in the presence of H_2O_2 and catalyzed by horseradish peroxidase (HRP). The resulting quinoneimine dye was measured at 520 nm (N-(4-antipyril)-3-chloro-5-sulfonate-p-benzoquinonemonoimine) (Fossati et al., 1980). Activity of ceruloplasmin (Cp) in the plasma was determined using a modified assay with p-phenylenediamine (Sunderman and Nomoto, 1970). Briefly 20 μL of plasma sample was diluted with 1.6 mL 0.4 M acetate buffer (adjusted to pH 5.5) and added to 0.2 mL freshly prepared buffered p-phenylenediamine solution (27.6 mmol/L) (SIGMA, Poznan, Poland), and then incubated at 37°C. The absorbance reflecting the intensity of the purple coloured product was measured by a spectrometer at 530 nm, after 5 min (blank) and 60 min (reaction) using 0.2 mL of sodium azide (1.5 mol/L) (SIGMA, Poznan, Poland) for stopping the enzyme reaction. The oxidase activity of Cp was expressed in U/L. Total glutathione (GSH+GSSG) was determined in the blood using a Total Glutathione Assay (Cell Biolabs, Inc., San Diego, USA). The method is based on reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) by glutathione reductase in the presence of NADPH. Subsequently, the chromogen reacts with the thiol group of GSH to produce a colored compound that absorbs at 405 nm. The total glutathione content in unknown samples is determined by comparison with the predetermined glutathione standard curve. The rate of chromophore production is proportional to the concentration of glutathione within the sample. The rate can be determined from the absorbance change over time. Metaphosphoric acid is provided to remove interfering proteins or enzymes from samples. Total antioxidant status (TAS) was determined using a diagnostic kit (Randox, Poland). Within the assay 2,2'-azino-di[3-ethylbenzthiazoline sulphonate (ABTS) is incubated with a peroxidase (metmyoglobin) and H_2O_2 to produce the radical cation ABTS. This has a relatively stable blue-green colour, which is measured at 600 nm. Antioxidants suppress colour production to a degree which is proportional to their concentration. Vitamin C content was determined using an ELISA kit (Cell Biolabs, Inc., San Diego, USA).

Redox status analysis in the tissues

As described in a previous work (Ognik and Wiertelcki, 2012), the following indicators of antioxidant status were determined in the homogenates from the rat organs: activity of superoxide dismutase (SOD) and catalase (CAT), and the concentration of lipid peroxides (LOOH), malondialdehyde (MDA), reduced glutathione (GSH) and vitamin C.

Redox status of protein and DNA, epigenetic changes and apoptotic assay in the brain

In the brain the content of protein carbonyl (PC) derivatives as an indicator of oxidation of amino acid residues and 8-hydroxydeoxyguanosine (8-OHdG) as a marker of oxidation of DNA bases were determined using OxiSelect diagnostic kits (Cell Biolabs, Inc., San Diego, USA). Briefly PC and 8-OHdG content was quantified using a standard curve by means of a competitive enzyme immunoassay. The

level of epigenetic changes in the brain of the rats was determined by analysing DNA methylation using diagnostic kits (cat. #MDQ1) manufactured by Sigma Aldrich. The methylated DNA was detected using the Capture and Detection antibodies, then quantified colorimetrically. The amount of methylated DNA present in the sample was proportional to the absorbance measured at 450 nm. DNA was isolated from the blood and brain using kits manufactured by QIAGEN. The content of caspase 8 (Casp 8) in the brain was determined using Caspase 8 ELISA Kit (Bioassay Technology Laboratory, Inc., China).

Statistical analysis

Data were checked for normality before statistical analysis was performed (the Shapiro-Wilk test). All data obtained were subjected to Student's *t*-test procedure and differences were considered significant at $P \leq 0.05$. In the tables, results are presented as mean values and the SEM (standard error of the mean; SEM is calculated as SD for all rats divided by the square root of rat number, $n=16$).

Results

Our study showed that feeding rats a diet lacking added Cu for 35 days had no effect on the relative weight of internal organs (Table 3). Lack of added copper in the rats diet caused a decrease of Cu content in the heart, lungs and testes ($P < 0.0001$, $P = 0.025$, $P = 0.021$ and $P = 0.037$, respectively; Table 4). In the blood of rats receiving a diet without the addition of Cu (group Cu-0), lower Cp activity ($P = 0.006$) and greater GPx and CAT activity ($P < 0.001$ and $P = 0.027$, respectively) were observed than in the rats whose diet included a copper supplement of 6.5 mg/kg (group Cu-6.5) (Table 5). The diet without added Cu resulted in increased lipid peroxidation, as evidenced by an increased LOOH level in the small intestine, liver, and heart ($P = 0.009$, $P = 0.008$ and $P < 0.001$, respectively; Table 6), as well as increased MDA content in the liver, spleen, lungs, brain and testes ($P = 0.004$, $P = 0.006$, $P = 0.045$, $P < 0.001$ and $P = 0.045$, respectively; Table 7) relative to the Cu-6.5 group. The Cu-0 treatment resulted in a decrease in SOD activity in the heart, lungs and testes of the rats ($P = 0.036$; $P = 0.049$ and $P = 0.034$, respectively; Table 8) and a decrease in CAT activity in the small intestine ($P = 0.029$; Table 9) relative to group Cu-6.5. In the brain and testes of rats from the Cu-0 treatment, lower content of GSH + GSSG ($P = 0.007$ and $P = 0.013$, respectively; Table 10) was observed than in rats from the Cu-6.5 treatment. Feeding rats a diet without added Cu had no effect on the content of vitamin C in any of the tissues tested (Table 11). Compared to rats from the Cu-6.5 treatment, the brain of rats from the Cu-0 treatment showed an increase in the content of protein carbonyls (PCs) ($P = 0.049$) and 8-OHdG ($P = 0.05$), in DNA methylation ($P = 0.039$), and in the level of caspase 8 ($P = 0.028$) (Table 12).

Table 3. Organosomatic index

Tissue	Experimental groups		SEM	P-value
	Cu-0	Cu-6.5		
Mean initial body weight (g)	135	135	0.001	0.258
Mean final body weight (g)	311	304	0.004	0.203
Liver (%)	4.193	4.196	0.105	0.836
Jejunum (%)	2.289	2.238	0.035	0.136
Spleen (%)	0.265	0.283	0.034	0.127
Heart (%)	0.254	0.277	0.019	0.148
Lung (%)	0.410	0.389	0.029	0.075
Kidney (%)	0.625	0.627	0.106	0.064
Brain (%)	0.592	0.616	0.103	0.307
Testes (%)	1.025	1.063	0.082	0.063

SEM = standard error of the mean.

Cu-0 – rats fed a diet without Cu supplementation; Cu-6.5 – rats fed a diet with the standard dose of Cu (6.5 mg per kg of diet from CuCO₃).

Table 4. Content of Cu in the selected rats organs (mg/kg)

Tissue	Experimental groups		SEM	P-value
	Cu-0	Cu-6.5		
Wall of jejunum	4.17	4.99	0.256	0.083
Spleen	2.18	2.53	0.346	0.078
Heart	1.15 b	2.37 a	0.668	0.025
Lung	2.49 b	3.23 a	0.764	0.021
Kidney	2.86	3.12	0.421	0.098
Testes	1.77 b	2.42 a	0.543	0.037
Quadriceps	3.05	3.46	0.486	0.088

a, b – means within the same row differ significantly ($P \leq 0.05$) according to Student's *t*-test procedure. SEM = standard error of the mean.

Table 5. Redox parameters in rat blood

Item	Experimental groups		SEM	P-value
	Cu-0	Cu-6.5		
LOOH $\mu\text{mol/L}$	64.83	62.56	0.035	0.356
MDA $\mu\text{mol/L}$	3.411	3.217	0.083	0.126
Cp U/L	11.21	12.90	0.008	0.006
GPx U/gHb	2052.2 a	1830.3 b	0.282	<0.001
CAT U/gHb	1976.5 a	1842.2 b	0.109	0.027
TAS mmol/L	1.086	0.988	0.098	0.209
GSH+GSSG $\mu\text{mol/L}$	0.161	0.159	0.018	0.725
Vit. C $\mu\text{mol/L}$	18.2	18.4	0.013	0.356

a, b – means within the same row differ significantly ($P \leq 0.05$) according to Student's *t*-test procedure. SEM = standard error of the mean.

LOOH – lipid peroxides; MDA – malondialdehyde; Cp – ceruloplasmin; GPx – glutathione peroxidase; CAT – catalase; TAS – total antioxidant status; GSH+GSSG – total glutathione; Vit. C – vitamin C.

Cu-0 – rats fed a diet without Cu supplementation; Cu-6.5 – rats fed a diet with the standard dose of Cu (6.5 mg per kg of diet from CuCO₃).

Table 6. Content of lipid peroxides (LOOH; $\mu\text{mol/kg}$) in rat tissues

Tissue	Experimental groups		SEM	P-value
	Cu-0	Cu-6.5		
Liver	1.796 a	1.250 b	0.025	0.009
Wall of jejunum	1.312 a	1.039 b	0.012	0.008
Spleen	1.245	1.261	0.018	0.245
Heart	1.373 a	0.951 b	0.032	<0.001
Lung	0.628	0.634	0.025	0.283
Kidney	1.651	1.456	0.026	0.625
Brain	4.247	4.211	0.328	0.085
Testes	3.423	3.507	0.023	0.104
Quadriceps	0.519	0.509	0.011	0.059

a, b – means within the same row differ significantly ($P \leq 0.05$) according to Student's *t*-test procedure. SEM = standard error of the mean.

Cu-0 – rats fed a diet without Cu supplementation; Cu-6.5 – rats fed a diet with the standard dose of Cu (6.5 mg per kg of diet from CuCO_3).

Table 7. Content of malondialdehyde (MDA; $\mu\text{mol/kg}$) in rat tissues

Tissue	Experimental groups		SEM	P-value
	Cu-0	Cu-6.5		
Liver	7.571 a	6.214 b	0.107	0.004
Wall of jejunum	19.06	19.58	0.095	0.933
Spleen	7.474 a	5.927 b	0.038	0.006
Heart	4.268	4.431	0.094	0.059
Lung	5.134 a	4.200 b	0.121	0.045
Kidney	7.671	7.849	0.109	0.125
Brain	9.945 a	4.879 b	0.025	<0.001
Testes	2.498 a	2.026 b	0.103	0.045
Quadriceps	5.383	5.650	0.135	0.185

a, b – means within the same row differ significantly ($P \leq 0.05$) according to Student's *t*-test procedure. SEM = standard error of the mean.

Cu-0 – rats fed a diet without Cu supplementation; Cu-6.5 – rats fed a diet with the standard dose of Cu (6.5 mg per kg of diet from CuCO_3).

Table 8. Activity of superoxide dismutase (SOD; U/g of protein) in rat tissues

Tissue	Experimental groups		SEM	P-value
	Cu-0	Cu-6.5		
Liver	1.950	1.930	0.126	0.109
Wall of jejunum	2.284	2.196	0.042	0.264
Spleen	2.153	1.948	0.025	0.064
Heart	1.940 b	2.141 a	0.013	0.036
Lung	2.071 b	2.320 a	0.026	0.049
Kidney	1.804	1.876	0.108	0.624
Brain	3.269	3.390	0.029	0.624
Testes	1.443 b	1.801 a	0.075	0.034
Quadriceps	1.724	1.683	0.038	0.069

a, b – means within the same row differ significantly ($P \leq 0.05$) according to Student's *t*-test procedure. SEM = standard error of the mean.

Cu-0 – rats fed a diet without Cu supplementation; Cu-6.5 – rats fed a diet with the standard dose of Cu (6.5 mg per kg of diet from CuCO_3).

Table 9. Activity of catalase (CAT; U/g of protein) in rat tissues

Tissue	Experimental groups		SEM	P-value
	Cu-0	Cu-6.5		
Liver	4.700	4.921	0.029	0.062
Wall of jejunum	13.03 b	15.73 a	0.108	0.029
Spleen	19.194	19.547	0.126	0.068
Heart	18.84	19.67	0.104	0.085
Lung	6.977	7.132	0.135	0.102
Kidney	17.24	16.84	0.035	0.345
Brain	10.30	10.96	0.027	0.344
Testes	6.964	7.152	0.086	0.685
Quadriceps	12.44	12.97	0.038	0.084

a, b – means within the same row differ significantly ($P \leq 0.05$) according to Student's *t*-test procedure. SEM = standard error of the mean.

Cu-0 – rats fed a diet without Cu supplementation; Cu-6.5 – rats fed a diet with the standard dose of Cu (6.5 mg per kg of diet from CuCO_3).

Table 10. Content of total glutathione (GSH+GSSG; $\mu\text{mol/kg}$) in rat tissues

Tissue	Experimental groups		SEM	P-value
	Cu-0	Cu-6.5		
Liver	0.195	0.181	0.012	0.195
Wall of jejunum	0.512	0.548	0.075	0.075
Spleen	1.417	1.393	0.084	0.108
Heart	0.078	0.077	0.008	0.088
Lung	0.258	0.253	0.013	0.204
Kidney	3.711	3.758	0.128	0.076
Brain	0.156 b	0.219 a	0.021	0.007
Testes	1.711 b	1.953 a	0.042	0.013
Quadriceps	0.147	0.150	0.007	0.124

a, b – means within the same row differ significantly ($P \leq 0.05$) according to Student's *t*-test procedure. SEM = standard error of the mean.

Cu-0 – rats fed a diet without Cu supplementation; Cu-6.5 – rats fed a diet with the standard dose of Cu (6.5 mg per kg of diet from CuCO_3).

Table 11. Content of vitamin C ($\mu\text{mol/kg}$) in rat tissues

Tissue	Experimental groups		SEM	P-value
	Cu-0	Cu-6.5		
Liver	36.00	34.06	0.382	0.264
Wall of jejunum	38.50	35.58	0.189	0.349
Spleen	7.944	7.931	0.082	0.104
Heart	87.76	84.70	0.219	0.073
Lung	69.43	68.13	0.142	0.077
Kidney	22.39	24.24	0.283	0.358
Brain	48.13	48.44	0.095	0.092
Testes	72.03	74.80	0.105	0.082
Quadriceps	34.38	33.08	0.102	0.142

SEM = standard error of the mean.

Cu-0 – rats fed a diet without Cu supplementation; Cu-6.5 – rats fed a diet with the standard dose of Cu (6.5 mg per kg of diet from CuCO_3).

Table 12. Redox parameters and epigenetic changes in brain of rats

Tissue	Experimental groups		SEM	P-value
	Cu-0	Cu-6.5		
PC nmol/mg protein	1.97 a	1.56 b	0.009	0.049
8-OHdG ng/g tissue	1.86 a	1.34 b	0.008	0.045
% methylation DNA	8.987 a	8.175 b	0.056	0.039
Casp 8 ng/mg tissue	1.26 a	0.89 b	0.013	0.028

a, b – means within the same row differ significantly ($P \leq 0.05$) according to Student's *t*-test procedure. SEM = standard error of the mean.

Cu-0 – rats fed a diet without Cu supplementation; Cu-6.5 – rats fed a diet with the standard dose of Cu (6.5 mg per kg of diet from CuCO_3).

Discussion

Our research shows that feeding rats a diet without the addition of copper increased the content of lipid oxidation products LOOH and MDA in the liver, small intestinal wall, heart, spleen, lungs, brain and testes. These results are unfavourable, as they indicate intensification of oxidative processes in these organs. Balevska et al. (1981) have also reported an increased LOOH level in the hepatic microsomes and mitochondria as a result of feeding rats a Cu-deficient diet. Results obtained by Chen et al. (1994) are also consistent with ours; the level of lipid oxidation products in the heart of rats receiving a Cu-deficient diet (0.4 $\mu\text{g/kg}$ diet) for four weeks increased two-fold relative to rats fed a diet with the recommended level of Cu (6 $\mu\text{g/g}$ diet). In these studies, the intensity of lipid oxidation processes in the organs was accompanied by a decrease in superoxide dismutase activity (Balevska et al., 1981; Chen et al., 1994). Likewise, our study found a reduction in the activity of this enzyme in the heart, lungs and testes. It can therefore be assumed that due to the lowering of the Cu level in these organs, the activity of antioxidant enzymes is inhibited, resulting in impairment of antioxidant defence. Increasing of the level of free radicals leads to escalating of the process of lipid peroxidation in the internal organs. However, it should be noted that in the liver, enhanced synthesis of LOOH and MDA was not accompanied by a decrease in SOD activity. This is very interesting because the level of copper in the liver significantly decreased as a result of giving the rats a diet with a reduced content of copper (Cholewińska et al., 2018 b). Activation of superoxide dismutase (SOD1) occurs *inter alia* in liver cells with the participation of copper chaperone for superoxide dismutase 1 (CCS), which includes Cu (I) in apoenzyme (Palumaa, 2013). Therefore, it may be assumed that the decrease in Cu in the liver occurred due to the consumption of a certain pool of this element for the synthesis of SOD. Presumably, however, the activity of antioxidants in the liver was too low in relation to the amount of free radicals formed, which is why the increased lipid peroxidation was observed in this organ.

Copper does not penetrate passively into the brain but is transported across the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCB) with the participation of special proteins such as CTR1, ATP7A and ATP7B (Telianidis et al.,

2013; Scheiber et al., 2014). Although the level of Cu in the brain of the Cu-0 group decreased only slightly relative to the Cu-6.5 group, in this experiment it was noted that the use of diet without added copper caused the greatest lipid oxidation in the brain among the organs tested and the increased oxidation of proteins and DNA. In our previous research on rats receiving a Cu-deficient diet (Ognik et al., 2019), we noted an increase in the plasma level of PCs, indicating increased protein oxidation. Protein carbonylation involves the introduction of carbonyl (aldehyde or ketone) groups to the protein structure. This usually takes place by direct oxidation of lysine, arginine, proline or threonine residues present in the protein chain (Ognik et al., 2019). Carbonylation of proteins is irreversible, and therefore they are not subject to repair processes. They can only be degraded by the proteasomal system (Höhn and Grune, 2014). Oxidative modifications of proteins in the body can negatively affect various cellular functions involving proteins such as receptors, signal transduction mechanisms, transport systems and enzymes. In addition, protein carbonyl derivatives can have a detrimental effect on other biomolecules, causing inactivation of DNA repair enzymes, the loss of specific properties of polymerase during DNA replication, and the development of new antigens on the surface of cells, inducing autoimmune reactions (Cakatay et al., 2001). Excessive production and accumulation of PCs in the body are believed to contribute to the development of a number of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis. In addition, PCs accumulate in the brain of multiple sclerosis patients (Jaiser and Winston, 2010). This suggests that brain proteins are particularly susceptible to carbonylation, confirming our observations. Nevertheless, in our previous research mentioned above (Ognik et al., 2019), we did not observe an increase in markers of lipid oxidation (MDA and LOOH) or DNA oxidation (8-OHdG) in the plasma of rats receiving a copper-deficient diet. 8-OHdG is the main product of oxidative DNA damage (Gürler et al., 2014). It is formed by the addition of a hydroxyl group at the 8th position of guanine (Ognik et al., 2019). Its quantity increases in Alzheimer's disease and during ageing, most likely due to decreased activity of antioxidant enzymes (Yanar et al., 2011). Based on the results of our previous studies (Cholewińska et al., 2018 b), in which the deficiency of copper in the diet caused a small, statistically non-significant reduction in the level of Cu in the brain, it may be assumed that this organ is very sensitive to the effects of free radicals. Although no significant reduction in the level of antioxidant enzymes in the brain of rats such as superoxide dismutase and catalase was observed, there was a significant decrease in the level of total glutathione, which is an endogenous antioxidant. Therefore, it may be assumed that the mechanisms of defending the brain against the action of free radicals are weakened with a reduced level of Cu in the body resulting in increased peroxidation of proteins and DNA.

In our study, feeding rats a diet without added copper also resulted in greater DNA cytosine methylation in the brain than in rats receiving a diet with the addition of 6.5 mg/kg Cu. DNA methylation plays a key role in the regulation of gene expression and in cell differentiation and development (Yang et al., 2015). In this process, DNA methyltransferases (Dnmts) catalyse the covalent attachment of a methyl group derived from S-adenylmethionine (SAM) to the fifth carbon of a cytosine

residue within CpG islands, resulting in the formation of 5-methylcytosine (Lisanti et al., 2013; Moore et al., 2013). Although DNA methylation is a naturally occurring process in the body, responsible, for example, for parental imprinting or silencing of proto-oncogenes, abnormalities in the DNA methylation pattern can have many adverse effects. The removal of methyl groups from CpG islands within proto-oncogene promoters may result in the expression of previously silenced genes and the onset of tumour development (Udomsinprasert et al., 2016). Abnormalities in DNA methylation occur in patients suffering from cancer and diabetes. They are also observed in other diseases, such as multiple sclerosis, autism, schizophrenia, Alzheimer's disease, stroke, and Parkinson's disease (Yang et al., 2015). Analysis of the results of our research indicates that the oxidative stress noted in the tissues of rats fed a diet without added Cu may be one of the factors disturbing the normal methylation pattern in the body (Gaetke and Chow, 2003; Venza et al., 2015). As a consequence, it may lead to the silencing of genes encoding important enzymes responsible for antioxidant defence and to increased production of free radicals, causing further DNA damage (Venza et al., 2015). Available literature proves that S-adenosylmethionine (SAM) is a donor of methyl groups in the DNA methylation process. SAM giving up the methyl group is transformed into S-adenosylhomocysteine (SAH). This, in turn, with the help of adenosylhomocysteinase (AHCY) in the presence of copper is reconstituted to SAM, which may further donate methyl groups. Nevertheless, too much copper may act as an inhibitor of AHCY, preventing the conversion of SAH into SAM and lowering the level of DNA methylation (Le et al., 2014). In our studies, we noted a higher level of DNA methylation in the Cu-0 group than Cu-6.5, which may be confirmed by these reports. As a result of excessive methylation of DNA, however, it may result in the process of methylation of important genes responsible for the expression of key proteins, including antioxidant enzymes.

The results of our research showed that feeding rats a diet without added copper reduced Cp levels in the blood. Similar results have been obtained by Prohaska and Lukasewycz (1981) and by Seol et al. (2015), who fed a Cu-deficient diet to mice. Ceruloplasmin is a plasma protein that can bind six copper atoms. About 95% of the copper present in plasma is bound to ceruloplasmin. This protein has the ability to oxidize both copper and iron in the process of reducing dioxygen to water, thus causing their mobilization in the body (Hellman and Gitlin, 2002; Jursa et al., 2008). As an acute phase protein, ceruloplasmin plays an important role in the fight against inflammation and also exhibits strong antioxidant properties (Dubick et al., 2015). The antioxidant properties of ceruloplasmin may result from the fact that as a ferroxidase it catalyses the oxidation of Fe^{2+} to Fe^{3+} , as well as from its ability to scavenge free radicals (Sirajwala et al., 2007). The results of our research may suggest that the amount of copper present in the plasma was insufficient to supply the copper ions needed to ensure proper function of ceruloplasmin. Our research shows that the activity of the antioxidant enzymes GPx and CAT in the blood was higher in the Cu-0 group than in the Cu-6.5 group. In contrast, in the liver of rats from the Cu-0 group, GPx and CAT activity were lower than in rats from the Cu-6.5 group. Glutathione (γ -L-glutamyl-L-cysteinyl-glycine) is the most common intracellular

thiol in most cells of living organisms. Glutathione can be synthesized in cells in a two-step enzymatic process dependent on ATP. The first step in glutathione synthesis is the conjugation of glutamic acid and cysteine, catalysed by glutamate-cysteine ligase, resulting in the formation of γ -glutamyl-cysteine (γ -Glu-Cys). In the second step, catalysed by glutathione synthase, glycine is added to γ -Glu-Cys, resulting in the formation of a glutathione molecule. Glutathione functions in the body as an antioxidant, constituting a cofactor or substrate for glutathione peroxidase and glutathione S-transferases (Yamada et al., 2018). Moreover, the reduced form of glutathione serves as an endogenous antioxidant which donates H^+ ions in the deactivation of reactive H_2O_2 (Weschawalit et al., 2017). The reaction is catalysed by GPx. There are eight glutathione peroxidases including cytosolic GPx1 and plasma GPx3 (Surai et al., 2018). The results of our research indicate that the Cu deficiency in the rat diet caused a decrease in the content of GSH in the brain and testes of rats relative to the control group, which may suggest that strong free radical reactions were taking place in these tissues, resulting in depletion of the pool of glutathione. The available literature indicates that disturbances of homeostasis in both the quantity of glutathione and the activity of glutathione-dependent enzymes may be associated with the induction and progression of numerous neurodegenerative diseases, such as Alzheimer's, Parkinson's and Huntington's diseases, amyotrophic lateral sclerosis, Friedreich's ataxia, Menkes and Wilson diseases (Johnson et al., 2012; Bhattacharjee et al., 2017). Apoptosis is a form of programmed cell death which is responsible for the spontaneous elimination of the cell when it has become irreversibly damaged or mutated. Apoptosis is therefore important in maintaining tissue homeostasis. This multi-step process involves proteases known as caspases (Carmody and Cotter, 2000). Gybina et al. (2009) report that deficiency of Cu in the body is accompanied by the release of cytochrome c from the mitochondrion to the cytosol, where it binds to the adapter protein Apaf-1 and pro-caspase-9. Next, this protein complex leads to the activation of 'executioner' caspases, such as caspase-3. Caspase-3, among other activated proteases, activates the proteolytic cascade leading to cell death. In addition, a significant reduction is then observed in the content of the protein Bcl-2, which is also released from the mitochondrion to the cytosol, with a completely opposite effect to that of cytochrome c – it prevents the activation of apoptosis. Abnormal apoptosis may be associated with the occurrence of neurodegenerative diseases such as Alzheimer's disease, amyotrophic lateral sclerosis and Huntington's disease, as well as ischaemic injury and several forms of retinal degeneration, collectively referred to as retinitis pigmentosa (RP) (Carmody and Cotter, 2000). The results of our research indicate that copper deficiency in the diet of rats contributes to increased activity of caspase 8 in the brain, which, based on our other results, suggests that increased production of free radicals damages the brain cells, which are then directed to the pathways of apoptotic cell death.

Conclusions

The research on rats has shown that a deficiency of Cu in the diet impairs the body's antioxidant defences, which in turn leads to increased lipid oxidation in the liver, small intestinal wall, heart, spleen, lungs, brain and testes, as well as to oxida-

tion of proteins and DNA in the brain. A deficiency of Cu in the diet also increases methylation of cytosine in the brain.

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