



ARE THE BIODISTRIBUTION AND METABOLIC EFFECTS OF COPPER NANOPARTICLES DEPENDENT ON DIFFERENCES IN THE PHYSIOLOGICAL FUNCTIONS OF DIETARY FIBRE?*

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Abstract

The aim of the study was to determine the effect of the recommended (6.5 mg/kg) or enhanced (13 mg/kg) level of CuNPs in the diet in combination with different types of dietary fibre – cellulose (control), inulin, pectin, or psyllium on the Cu biodistribution and level of selected minerals parameters in the blood of rats. Rats were randomly divided into 10 groups. The first two groups were fed control diets containing cellulose and a mineral mixture with standard or enhanced content of CuCO₃. Experimental groups were fed a diet supplemented with CuNPs (6.5 or 13 mg/kg) and combined with different types of fibre (cellulose, pectin, inulin, or psyllium). After the feeding period blood, liver, brain, and thigh samples were collected. In the samples of water, diet, urine, faeces, liver, brain, and thigh the Cu content was determined to assess Cu biodistribution in the body. Additionally, the concentrations of minerals (Cu, P, Ca, Mg, Fe, and Zn) in the blood plasma samples were measured. The replacement of CuCO₃ with CuNPs in the diet beneficially influenced the biodistribution of Cu in the body by reducing its excretion, improving its digestibility, and utilization, reducing its accumulation in the brain and muscle, and increasing levels of Ca, P, Mg, Zn and Fe in the blood. Increasing the level of CuNPs in the diet increased total Cu intake. The addition of pectin, inulin and psyllium to the diet with a high content of CuNPs significantly increased the excretion of Cu, with no negative effect on its digestibility, and utilization, and prevented its excessive accumulation in the brain and muscle of rats, especially in the case of inulin. The results suggest that the addition of dietary fibre to the diet of rats ensures homeostasis of this element in the case of excessive intake of CuNPs by modifying the bioavailability of Cu.

Key words: copper nanoparticles, dietary fibre, rat, biodistribution, mineral indices

The presence of copper in the body is essential because it guarantees the correct course of many metabolic processes, such as cellular respiration, synthesis of neurotransmitters, blood clotting, iron metabolism, haemoglobin synthesis, antioxidant defence, and immune responses, particularly as a cofactor of numerous enzymes (Brewer, 2010; Angelova et al., 2011; Opazo et al., 2014; Kumar et al., 2015, 2016; Bost et al., 2016; Tishchenko et al., 2016; Ognik et al., 2016). Cu deficiency in the diet can therefore result in a number of pathological changes in the functioning of the body (Ognik et al., 2016). The literature shows that it can be associated with an increased risk of neurodegenerative diseases such as Parkinson's disease, demyelination of the brain, or optic neuropathy (Jaiser and Winston, 2010), as well as intestinal resorption disorders (Lorincz, 2018) or problems with iron absorption and metabolism resulting in anaemia (Sawosz et al., 2018). Although Cu deficiencies are relatively rare, in some cases additional enrichment of the diet with this microelement is required. This takes place

in particular in people with a genetic defect leading to the development of Menkes disease, manifested as impaired Cu absorption and thus a reduced level of Cu in the body, as well as in animals whose diet is insufficiently varied, with low levels of ingredients containing Cu (Ognik et al., 2016). To increase the level of Cu in the body, the diet is most often supplemented with inorganic Cu compounds such as sulphate or carbonate, which, however, are poorly absorbed (Ognik et al., 2016; Sawosz et al., 2018; Scott et al., 2018; Cholewińska et al., 2018 a). Our own previous research (Cholewińska et al., 2018 a), as well as studies by other authors (Lee et al., 2016 a; Sawosz et al., 2018), show that the bioavailability of Cu can be significantly increased by replacing inorganic forms of Cu with Cu nanoparticles (CuNPs). CuNPs have a number of beneficial properties that facilitate their absorption and utilization in the body, such as their small size but large active surface or the lack of an electric charge (Lee et al., 2016 a; Ognik et al., 2016; Cholewińska et al., 2018 b).

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Although Cu absorption begins in the stomach, where inorganic compounds dissociate to Cu^+ and Cu^{2+} ions in the acidic environment, this process is most efficient in the first part of the small intestine, i.e. the duodenum and early jejunum (Kim et al., 2008; Ognik et al., 2016; Lee et al., 2016 a). Cu from chyme located in the small intestinal lumen enters the enterocytes by active transport involving the proteins DMT1 (divalent metal transporter 1) and CTR1 (copper transporter 1) or by endocytosis or pinocytosis. It is then transported from the enterocytes to the blood by the protein ATP7A (Sharp, 2003; Ognik et al., 2016). It has been demonstrated that a certain portion of ingested CuNPs can dissociate to Cu^{2+} ions in the low pH of the stomach, allowing them to be absorbed by the same mechanisms as inorganic copper compounds. Due to their very small size, however, CuNPs can also passively penetrate cells through pores in the cell membrane or undergo intercellular passage (Ognik et al., 2016).

After Cu enters the bloodstream from the intestinal lumen, portal circulation first transports it to the liver, which is the most important organ for its metabolism and bio-distribution. Here a significant portion of Cu is bound to ceruloplasmin produced by the hepatocytes, and in this complex, it is released to the peripheral blood, which distributes it to the other tissues. Free ionic Cu in the peripheral blood can combine with other plasma proteins, such as albumin or metallothionein (Wijmenga and Klomp, 2004). Cu which has reached the liver can also accumulate in it, and the amount of accumulated Cu increases with its level in the diet (Ognik et al., 2016; Cholewińska et al., 2018 a). Apart from the liver, the brain, kidneys, heart, and skeletal muscles have also been shown to have a tendency to accumulate ionic forms of copper (Gibson, 2005; Lee et al., 2016 a), and in the case of CuNPs, the spleen and lungs as well (Lee et al., 2016 a, b).

It is estimated that only 20% of the total Cu absorbed from the gastrointestinal tract undergoes excretion processes. The predominant route of excretion of Cu from the body is binding with bile in the liver, during which water-insoluble complexes are formed. Then they are released into the intestines and excreted in the faeces. Only 2% of Cu is eliminated from the body in the urine (Wijmenga and Klomp, 2004).

Our previous research showed increased bioavailability of CuNPs relative to inorganic copper compounds, as indicated by a decrease in their excretion in the urine and faeces as well as an increase in the Cu utilization index. On the other hand, increasing the level of CuNPs in the diet of rats increased the excretion of this microelement and reduced its utilization in the body (Cholewińska et al., 2018 a). An excessive level of Cu may be linked to a number of pathological changes leading to numerous serious illnesses (Bost et al., 2016). The toxicity of Cu is primarily associated with the fact that as a transition metal, it undergoes the Fenton and Haber–Weiss reactions in the body, resulting in increased synthesis of free radicals. This in turn increases oxidative stress, which leads to the development of metabolic syndrome, a number of neu-

rodegenerative disorders, and even cancers (Bost et al., 2016; Ognik et al., 2018 a, 2020). Due to the increased bioavailability of CuNPs, Cu levels high enough to induce a toxic effect seem to be attained even more easily than in the case of its inorganic forms. According to Coudray et al. (2003) and Wijmenga and Klomp (2004), increased excretion of Cu may be one of the most important mechanisms enabling the maintenance of its homeostasis in the body, which seems fully consistent with the findings of our previous research (Cholewińska et al. 2018 a, b).

The available literature indicates that the degree of absorption of microelements, including Cu, can be modified not only by their homeostasis in the body but also by other nutrients in the diet, including dietary fibre (Gralak et al., 1996; Kim and Shin, 1996; El-Zoghbi and Sitohy, 2001; Coudray et al., 2006; Krzysik et al., 2009). Enrichment of the diet with dietary fibre unquestionably has a number of advantages, as it stimulates peristalsis, enhances detoxification processes, and helps to maintain a normal body weight (Kieffer et al., 2016; Maćkowiak et al., 2016). Nevertheless, there are reports indicating that dietary fibre exhibits a tendency to inhibit the absorption of microelements present in the ingesta (Rockway et al., 1987; Caballero, 1988; Adams et al., 2018). This may be due to the fact that increasing peristalsis, it reduces the time the ingesta remains in the intestine, thereby decreasing the absorption of elements from it (Caballero, 1988; Spiller, 2001; Krzysik et al., 2009). Moreover, owing to the presence of free carboxyl groups, water-soluble dietary fibre, which forms a viscous gel in the digestive tract, has been shown to bind divalent ions, including Cu. The resulting complexes are unable to penetrate the intestinal mucosa and thus are excreted in the faeces, thereby reducing the bioavailability of Cu (Krzysik et al., 2009; Baye et al., 2017; Capuano, 2017). The results of our previous research also indicate that the addition of dietary fibre in the form of pectin, inulin, or psyllium strengthens the intestinal barrier, which can significantly affect the amount of Cu absorbed (Cholewińska et al., 2023).

Copper nanoparticles (CuNPs) are often chosen over other forms of copper for biodistribution studies due to their unique properties. CuNPs have higher bioavailability compared to other forms of copper, such as ions or micro-particles (Lee et al., 2016 a, b; Cholewińska et al., 2018 a). This is due to their small size, large surface area and high reactivity, which makes them easier to absorb and distribute throughout the body (Chudobova et al., 2015; Cholewińska et al., 2018 a). Our previous studies have shown that CuNPs are absorbed from the intestine to a greater extent than copper salts and excreted from the body to a lesser extent in faeces/urine (Cholewińska et al., 2018 a). Replacing copper carbonate with CuNPs also beneficially protected rat proteins and DNA against excessive oxidation processes (Ognik et al., 2018 b). However, it should be remembered that CuNPs may have toxicological effects, especially at higher doses. According to the results of our previous studies, CuNPs accumulated to a greater extent in the brain, showed a stronger antibacterial effect in the large intestine and inten-

sified lipid oxidation processes, causing the greatest damage to liver and lung tissue (Cholewińska et al., 2018 a, Ognik et al., 2018 b, 2020). Taking into account the growing interest in including CuNPs in the diet of animals and the wide range of diverse physiological effects of CuNPs throughout the body, it seems appropriate to thoroughly understand and be able to regulate the sites of nanoparticles' impact. There is no doubt that the beginning of this entire process is what happens in the intestine, i.e. the place of absorption that determines the amounts of nutrients and non-nutrients absorbed into the body. One of the most important components of the diet is dietary fibre, and its type will mainly determine the degree of absorption and, consequently, the physiological activity of copper nanoparticles. It has been proven that including dietary fibre in a diet containing CuNPs results in the interaction of these two ingredients, which may have a protective effect on the body. In our previous studies, we have shown that dietary fibre supplementation in the form of cellulose, inulin, pectin or psyllium can protect the small intestine against the potentially harmful oxidative effects of CuNPs by strengthening the intestinal barrier and limiting the absorption of CuNPs (Cholewińska et al., 2023). In light of the above, the choice of CuNPs as one of the experimental factors for testing the biodistribution of this ingredient in the body was dictated by two reasons. First, this was due to the nature of our previous studies using CuNPs, in which intermediate sizes were selected from a range of nanoparticle sizes from very small 2 nm to large 100 nm. Secondly, the choice of copper in the form of nanoparticles for research was dictated by the need to gain new knowledge whether there is a potential possibility of supporting their "beneficial face" and weakening the "dangerous face" by including various forms of dietary fibre in the diet.

Therefore we postulated that a dietary combination of CuNPs with various sources of dietary fibre – neutral cellulose (control), prebiotic inulin, viscous pectin, or swelling psyllium – influences the biodistribution of copper, thereby regulating the metabolic effect of CuNPs in the body. The aim of the study was to verify this hypothesis by assessing the effect of the inclusion of CuNPs in the diet of rats at the recommended level (6.5 mg Cu/kg diet) or double that level (13.0 mg Cu/kg diet) in combination with various sources of dietary fibre – cellulose, pectin, inulin or psyllium – on the biodistribution of Cu, by analysing the intake, excretion, digestibility, utilization, and distribution of Cu in the tissues, as well as levels of selected minerals in the blood.

Material and methods

Copper nanoparticles and dietary fibre

Copper nanoparticles with 99.9% purity were obtained from SkySpring Nanomaterials, Inc. (Houston, TX, USA). The experiment was carried out using the same CuNPs as those used in our previous studies (Cholewińska et al., 2018 a, b; Ognik et al., 2018 b, 2020; Cholewińska et al., 2022, 2023). Their physicochemical properties were examined in detail and described by Cholewińska et al. (2018 a). CuCO₃ used as a control dietary source of copper was purchased in Merck KGaA (Darmstadt, Germany). α -cellulose was used as a control dietary fibre source (Sigma, Poznań, Poland), and the following experimental dietary fibre sources were used: pectin (PectinE 440(I), Brouwland, Beverlo, Belgium), inulin (Frutafit Tex, Sensus, Netherlands), and psyllium (Psyllium husk powder, NaturaleBio, Rome, Italy).

Table 1. Composition of experimental diets administered to rats for 6 weeks

	C	CH	CN	CNH	PN	PNH	JN	JNH	SN	SNH
Casein ¹	14.8	14.8	14.8	14.8	14.8	14.8	14.8	14.8	14.8	14.8
DL-methionine	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Cellulose ²	8.0	8.0	8.0	8.0	2.0	2.0	2.0	2.0	2.0	2.0
Pectin					6	6				
Inulin							6	6		
Psyllium									6	6
Choline chloride	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Rapeseed oil	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
Cholesterol	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Vitamin mix ³	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Mineral mix ⁴	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Maize starch ⁵	64.0	64.0	64.0	64.0	64.0	64.0	64.0	64.0	64.0	64.0
Calculation										
Cu from (mg/kg)										
CuCO ₃	6.5	13	0	0	0	0	0	0	0	0
CuNPs	0	0	6.5	13	6.5	13	6.5	13	6.5	13

¹Casein preparation: crude protein 89.7%, crude fat 0.3%, ash 2.0%, water 8.0%. ² α -Cellulose (SIGMA, Poznań, Poland), main source of dietary fibre. ³AIN-93G-VM (Reeves, 1997), 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⁻¹), 0.8 vitamin A (all-trans-retinyl palmitate, 500 000 IU/g), 0.25 vitamin D₃ (cholecalciferol, 400 000 IU g⁻¹), 0.075 vitamin K₁ (phyloquinone), 974.655 powdered sucrose. ⁴In the experimental treatments with CuNPs the MX did not include CuCO₃, and for the safety of the technician preparing the experimental diets, the CuNP preparation was added as an emulsion together with dietary rapeseed oil. This procedure has been successfully used in our previous experiments. ⁵Maize starch preparation: crude protein 0.6%, crude fat 0.9%, ash 0.2%, total dietary fibre 0%, water 8.8%.

Experimental protocol

The animals were obtained from the company's own breeding of laboratory rats (breeder register 051, Institute of Animal Reproduction and Food Research PAS, Olsztyn, Poland). Healthy 9-week-old outbred male Wistar rats (Cmdb:Wi CMDb) were fed for 6 weeks on a standard semi-purified rat diet with two levels of CuNPs (the recommended level and double that level, i.e. 6.5 and 13 mg/kg diet, respectively) in combination with different types of dietary fibre. All diets have been prepared in our laboratory using high-end ingredients, including casein as the main protein source, rapeseed oil as a fat source and maize starch as the main energy source (Table 1). The control diet contained a mineral mixture with a standard and high content of CuCO_3 (6.5 and 13 mg/kg diet). In the diets with CuNPs, the mineral mixture did not contain CuCO_3 and the copper nanoparticles were added to the diet together with rapeseed oil (as an emulsion) for the safety of the technician. The control dietary fibre, α -cellulose, was added at 8% of the diet, while the experimental fibres (inulin with a prebiotic effect, psyllium with a bulking effect, and pectin with a viscous effect) were added at 6% of the diet in place of cellulose. The experimental protocol consisted of 10 groups, $n = 10$ per group. All animal care and experimental procedures were in compliance with Polish legislation concerning animal experimentation and ethical practice, 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 approved by the Local Ethics Committee for Animal Experiments in Olsztyn (Approval No. 19/2021; Olsztyn, Poland).

Sample collection and analyses

During the study, the digestibility and utilization tests (balance tests) of copper (Cu) were carried out. Due to the large number of experimental groups and the limited number of metabolic cages, the balance test was carried out on only 6 individuals from each experimental group. All other physiological measurements were performed separately for all animals in each experimental group ($n=10$ for each group). After a 10-day preliminary period, faeces and urine were thoroughly collected for 5 days from rats that were kept in balance cages (Tecniplast SpA, Buguggiate, Italy). The content of Cu in diets, drinking water, faeces and urine collected in the balance period was assayed using the methods described below ($n=6$). The body weight of each animal was monitored at the start and termination of the experiment, while diet (the entire feeding period) and tap water (the balance test period) consumptions were checked daily. Before the end of the experiment, the rats were deprived of feed for 8 h but had free access to water. Next, they were anaesthetized *i.p.* with ketamine (K) and xylazine (X) in 0.9% NaCl (100 and 10 mg/kg BW, respectively) according to the anaesthesia and euthanasia guidelines for

laboratory rodents. Following laparotomy, blood samples were drawn from the caudal vena cava into heparinized tubes, and finally, the rats were euthanized by cervical dislocation ($n=10$). Then the liver, brain, and thigh were removed and weighed ($n=10$). The blood plasma was prepared by solidification and low-speed centrifugation (350 g, 10 min, 4°C). Plasma samples were kept frozen at -70°C until assay.

Sample preparation

Samples of water, diet, urine, faeces, plasma, liver, brain, and thigh were mineralized using microwave mineralizer (MARSXpress, CEM, Matthews, NC, USA). 4 mL of concentrated HNO_3 Suprapure (Merck KGaA, Darmstadt, Germany) was added to approximately 0.5 g of sample and digested using microwaves.

Copper analyses

The copper content in the samples of water, diet, urine, faeces, liver, brain, and thigh was determined by inductively coupled plasma-mass spectrometry (ICP-MS) with Varian 820-MS ICP Mass Spectrometer (Varian Analytical Instruments, Victoria, Australia) in order to assess Cu biodistribution in the body (Chen et al., 2007). The certified reference material NIST-1577C Bovine liver (Merck KGaA, Darmstadt, Germany) was used for quality control.

Analysis of plasma mineral concentrations

The concentrations of minerals (Cu, P, Ca, Mg, Fe, and Zn) in the blood plasma samples were determined by flame atomic absorption spectrometry (FAAS) with a Perkin-Elmer M 5000 atomic absorption spectrometer coupled with an HGA 500 graphite furnace (Perkin-Elmer Life and Analytical Sciences Co., Shelton, CT, USA).

Statistical analysis

STATISTICA software, version 12.0 (StatSoft Corp., Kraków, Poland), was used to determine whether variables differed among treatment groups. Two-way ANOVA was used to assess the effects of the main factors, i.e. CuNP inclusion level (L, 6.5 mg/kg and H, 13 mg/kg) and dietary fibre type (cellulose, pectin, inulin and psyllium), as well as the interactions between them. When the ANOVA indicated significant treatment effects, means were evaluated using Duncan's multiple-range test. In addition, each experimental group receiving the lower level of CuNPs (L) was compared with the control group C (fed a diet with 6.5 mg/kg Cu from CuCO_3 and cellulose as the main dietary fibre source) using a t-test. Similarly, the t-test was used to compare the experimental groups fed diets with the higher level of CuNPs (H) with the control group CH fed a diet with 13 mg/kg Cu from CuCO_3 and cellulose as the main dietary fibre source. Data were checked for normality prior to the statistical analysis. Differences at $P \leq 0.05$ were considered to be significant.

Table 2. Copper biodistribution balance test in rats fed experimental diets (n = 6 per group)*

		Cu intake from diet		Total Cu intake ^s		Urinal Cu		Faecal Cu		Total excreted Cu		Cu digestibility		Cu utilization		Brain Cu		Liver Cu		Thigh Cu	
		mg/5 d	mg/5 d	mg/5 d	mg/5 d	mg/5 d	mg/5 d	mg/5 d	mg/5 d	mg/5 d	mg/5 d	%	%	%	%	mg/kg	mg/kg	mg/kg	mg/kg		
Control C																					
Control CH		1.22	0.629	1.22	0.631	0.021	0.028	0.907	0.577	0.928	0.605	8.38	25.8	4.08	2.21	4.50	1.02	0.791			
2-way ANOVA																					
CN		0.618 c	0.618 c	0.620 cd	0.620 cd	0.017 de	0.017 de	0.478 c#	0.478 c#	0.496 c#	0.496 c#	22.8 c#	19.9 cd#	1.79#	4.10	0.714 cd#	0.714 cd#				
CNH		1.17 a	1.17 a	1.17 a	1.17 a	0.015 e	0.015 e	0.492 c&	0.492 c&	0.507 bc&	0.507 bc&	58.0 a&	56.8 a&	1.74	4.18	0.801 c	0.801 c				
PN		0.609 c	0.609 c	0.611	0.611	0.028 cde	0.028 cde	0.467 c#	0.467 c#	0.494 c#	0.494 c#	23.7 c#	19.1 cd#	1.71#	4.53	0.564 de#	0.564 de#				
PNH		1.07 b&	1.07 b&	1.07 b&	1.07 b&	0.023 cde	0.023 cde	0.846 a	0.846 a	0.870 a	0.870 a	20.6 c	18.4 cd	1.63	4.61	0.659 cde	0.659 cde				
JN		0.556 c	0.556 c	0.558 d	0.558 d	0.034 cd	0.034 cd	0.353 d#	0.353 d#	0.387 d#	0.387 d#	36.6 b#	30.6 b#	1.63#	4.26	0.457 e#	0.457 e#				
JNH		1.07 b&	1.07 b&	1.07 b&	1.07 b&	0.061 a&	0.061 a&	0.722 b	0.722 b	0.784 a	0.784 a	32.4 b	26.5 bc	1.66	4.19	1.13 b&	1.13 b&				
SN		0.685 c	0.685 c	0.687 c	0.687 c	0.051 ab	0.051 ab	0.548 c	0.548 c	0.599 b	0.599 b	19.9 c#	12.6 d#	1.64#	4.24	1.26 ab	1.26 ab				
SNH		1.06 b&	1.06 b&	1.06 b&	1.06 b&	0.040 bc&	0.040 bc&	0.822 a	0.822 a	0.861 a	0.861 a	22.6 c	18.7 cd	1.60	4.75&	1.33 a&	1.33 a&				
SEM		0.034	0.034	0.034	0.034	0.002	0.002	0.026	0.026	0.027	0.027	1.916	1.995	0.032	0.049	0.041	0.041				
CuNP dose (D)																					
L (6.5 mg/kg)		0.613 b	0.613 b	0.615 b	0.615 b	0.032	0.032	0.461	0.461	0.494	0.494	25.3	20.1	1.69	4.28	0.748	0.748				
H (13 mg/kg)		1.09 a	1.09 a	1.09 a	1.09 a	0.035	0.035	0.720	0.720	0.755	0.755	33.4	30.1	1.66	4.43	0.979	0.979				
P value		<0.001	<0.001	<0.001	<0.001	0.575	0.575	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.465	0.147	<0.001	<0.001				
Fibre type (F)																					
C (cellulose)		0.896	0.896	0.897	0.897	0.016	0.016	0.485	0.485	0.501	0.501	40.4	38.4	1.76	4.14c	0.758	0.758				
P (pectin)		0.838	0.838	0.840	0.840	0.025	0.025	0.656	0.656	0.682	0.682	22.1	18.8	1.67	4.57a	0.612	0.612				
J (inulin)		0.811	0.811	0.813	0.813	0.047	0.047	0.537	0.537	0.585	0.585	34.5	28.6	1.64	4.22bc	0.792	0.792				
S (psyllium)		0.872	0.872	0.873	0.873	0.045	0.045	0.684	0.684	0.730	0.730	21.2	15.7	1.62	4.50ab	1.29	1.29				
P value		0.058	0.058	0.058	0.058	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.237	0.009	<0.001	<0.001				
Interaction D×F																					
P value		0.061	0.061	0.062	0.062	0.006	0.006	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.867	0.202	<0.001	<0.001				

*The dietary treatments used in the experimental feeding period: groups C and CH, fed a control diet with standard and enhanced Cu content in the mineral mixture (6.5 and 13 mg/kg from CuCO₃, respectively), with 8% cellulose as dietary fibre source; groups CN and CNH, fed diets supplemented with CuNPs (6.5 and 13 mg/kg from Cu nanoparticles, respectively), with 8% cellulose as dietary fibre source; groups PN and PNH, fed diets supplemented with CuNPs (6.5 and 13 mg/kg from Cu nanoparticles, respectively), with 2% cellulose and 6% inulin as dietary fibre source; groups SN and SNH, fed diets supplemented with CuNPs (6.5 and 13 mg/kg from Cu nanoparticles, respectively), with 2% cellulose and 6% psyllium as dietary fibre source; L, treatment (n=24) with dietary CuNPs at 6.5 mg/kg diet; H, treatment (n=24) with dietary CuNPs at 13 mg/kg diet; C, treatment (n=12), with cellulose as dietary fibre; P, treatment (n=12), with inulin as dietary fibre; S, treatment (n=12) with psyllium as dietary fibre; a, b – mean values within a column with different letters are significantly different (P<0.05); differences between groups (CN, CNH, PN, PNH, JN, JNH, SN, SNH) are indicated with superscripts only in the case of a statistically significant interaction D×F (P<0.05). Additionally, each experimental group fed CuNPs at 6.5 mg/kg (CN, PN, JN, SN) was compared with the control C using a t-test (# indicates a significant difference vs group C); similarly, each experimental group fed CuNPs at 13 mg/kg (CNH, PNH, JNH, SNH) was compared with the control CH using a t-test (& indicates a significant difference vs group CH); BW, body weight; SEM, pooled standard error of mean (standard deviation for all rats divided by the square root of rat number, n=60).[†]Total Cu intake from diet and water (water Cu concentration was 0.0182 mg/L). Analysed dietary concentration of Cu in diets C, CH, CN, CNH, PN, PNH, JN, JNH, SN, SNH, 7.32, 14.8, 7.33, 13.33, 12.4, 7.23, 13.9, 8.40, 13.5 mg/kg, respectively.

Table 3. Blood plasma mineral concentrations in rats fed experimental diets (n=10 per group)*

	Ca	P	Mg	Cu	Zn	Fe
	mmol/L	mmol/L	mmol/L	μmol/L	μmol/L	μmol/L
Control C	1.82	3.43	0.304	128	16.6	9.11
Control CH	1.89	3.38	0.304	106	17.7	10.7
2-way ANOVA:						
CN	2.02 c#	5.90 #	0.338 b#	107 a#	18.9 e#	13.9 c#
CNH	2.09 bc&	5.51 &	0.394 a&	111 a&	20.1 d&	14.6 bc&
PN	2.17 b#	4.87 #	0.341 b#	101 b#	21.7 c#	10.9 d#
PNH	2.38 a&	4.86 &	0.338 b&	93.1 c&	21.6 c&	15.8 ab&
JN	2.43 a#	11.3	0.329 b#	90.4 c#	22.5 bc#	16.7 a#
JNH	2.12 b	4.72	0.338 b	98.1 b	23.2 b	16.5 a
SN	2.02 c#	5.08 #	0.332 b#	89.9 c#	24.3 a#	14.1 c#
SNH	2.01 c	5.01	0.325 b	90.7 c	23.4 ab	17.3 a
SEM	0.023	0.695	0.003	1.405	0.293	0.352
CuNP dose (D)						
L (6.5 mg/kg)	2.16	6.78	0.335	97.0	21.8	13.9
H (13 mg/kg)	2.15	5.03	0.349	98.3	22.1	16.1
P value	0.861	0.315	<0.001	0.254	0.298	<0.001
Fibre type (F)						
C (cellulose)	2.05	5.71	0.366	109	19.5	14.3
P (pectin)	2.27	4.86	0.340	96.9	21.7	13.3
J (inulin)	2.28	8.00	0.333	94.3	22.8	16.6
S (psyllium)	2.02	5.04	0.328	90.3	23.9	15.7
P value	<0.001	0.558	<0.001	<0.001	<0.001	<0.001
Interaction D×F						
P value	<0.001	0.469	<0.005	<0.001	0.024	<0.001

*The dietary treatments used in the experimental feeding period: groups C and CH, fed a control diet with standard and enhanced Cu content in the mineral mixture (6.5 and 13 mg/kg from CuCO₃, respectively), with 8% cellulose as dietary fibre source; groups CN and CNH, fed diets supplemented with CuNPs (6.5 and 13 mg/kg from Cu nanoparticles, respectively), with 8% cellulose as dietary fibre source; groups PN and PNH, fed diets supplemented with CuNPs (6.5 and 13 mg/kg from Cu nanoparticles, respectively), with 2% cellulose and 6% pectin as dietary fibre source; groups JN and JNH, fed diets supplemented with CuNPs (6.5 and 13 mg/kg from Cu nanoparticles, respectively), with 2% cellulose and 6% inulin as dietary fibre source; groups SN and SNH, fed diets supplemented with CuNPs (6.5 and 13 mg/kg from Cu nanoparticles, respectively), with 2% cellulose and 6% psyllium as dietary fibre source; L, treatment (n=40) with dietary CuNPs at 6.5 mg/kg diet; H, treatment (n=40) with dietary CuNPs at 13 mg/kg diet; C, treatment (n=20), with cellulose as dietary fibre; P, treatment (n=20), with cellulose and 6% pectin as dietary fibre; J, treatment (n=20), with inulin as dietary fibre; S, treatment (n=20) with psyllium as dietary fibre; a, b – mean values within a column with different letters are significantly different (P<0.05); differences between groups (CN, CNH, PN, PNH, JN, JNH, SN, SNH) are indicated with superscripts only in the case of a statistically significant interaction D×F (P<0.05). Additionally, each experimental group fed CuNPs at 6.5 mg/kg (CN, PN, JN, SN) was compared with the control C using a t-test (# indicates a significant difference vs group C); similarly, each experimental group fed CuNPs at 13 mg/kg (CNH, JNH, SNH) was compared with the control CH using a t-test (& indicates a significant difference vs group CH); BW, body weight; SEM, pooled standard error of mean (standard deviation for all rats divided by the square root of rat number, n=100).

Results

One-way ANOVA

When describing the obtained results, the following designations of individual experimental groups were used: C – group fed a control diet with standard Cu content in the mineral mixture (6.5 mg/kg from CuCO_3), with 8% cellulose as dietary fibre source; CH – group fed control diet with enhanced Cu content in the mineral mixture (13 mg/kg from CuCO_3), with 8% cellulose as dietary fibre source; CN – group fed diets supplemented with CuNPs (6.5 mg/kg from Cu nanoparticles), with 8% cellulose as dietary fibre source; CNH – group fed diets supplemented with CuNPs (13 mg/kg from Cu nanoparticles), with 8% cellulose as dietary fibre source; PN – group fed diets supplemented with CuNPs (6.5 mg/kg from Cu nanoparticles), with 2% cellulose and 6% pectin as dietary fibre source; PNH – group fed diets supplemented with CuNPs (13 mg/kg from Cu nanoparticles), with 2% cellulose and 6% pectin as dietary fibre source; JN – group fed diets supplemented with CuNPs (6.5 mg/kg from Cu nanoparticles), with 2% cellulose and 6% inulin as dietary fibre source; JNH – group fed diets supplemented with CuNPs (13 mg/kg from Cu nanoparticles), with 2% cellulose and 6% inulin as dietary fibre source; SN – group fed diets supplemented with CuNPs (6.5 mg/kg from Cu nanoparticles), with 2% cellulose and 6% psyllium as dietary fibre source; SNH – group fed diets supplemented with CuNPs (13 mg/kg from Cu nanoparticles), with 2% cellulose and 6% psyllium as dietary fibre source.

One-way ANOVA for comparisons between experimental groups CN, PN, JN, and SN and the control group C at $P < 0.05$ showed a decrease in Cu excretion in the rats from groups CN, PN and JN relative to group C, particularly in the faeces. In comparison to group C, in all experimental groups – CN, PN, JN and SN – there was an increase in the Cu digestibility and utilization indices. Lower Cu content was also noted in the brain of rats from experimental groups CN, PN, JN and SN, while a decrease in the Cu level in the muscle was noted only in groups CN, PN and JN (Table 2). The plasma of rats from all experimental groups – CN, PN, JN and SN – also had higher levels of Ca, Mg, Zn and Fe, with a lower level of Cu, in comparison to group C. In addition, the plasma of rats from experimental groups CN, PN and SN had a higher level of P than in the rats from group C (Table 3).

One-way ANOVA for comparisons between experimental groups CNH, PNH, JNH and SNH and the control group CH at $P < 0.05$ showed a reduction in Cu intake in the rats from groups PNH, JNH and SNH relative to group CH. The analysis also revealed greater excretion of Cu in the urine in groups JNH and SNH than in group CH. The rats from group CNH showed a significantly lower level of Cu excretion, particularly in the faeces, as well as higher Cu digestibility and utilization indices than in group CH. The Cu level in the liver of rats from group SNH was higher than in group CH, while the Cu

level in the muscle was higher in both group SNH and JNH (Table 2). The plasma level of Cu was higher in group CNH and lower in group PNH than in group CH. The blood of rats from groups CNH and PNH also had significantly higher levels of Ca, P, Mg, Zn and Fe than the rats from group CH (Table 3).

Two-way ANOVA

Two-way ANOVA showed that increasing the level of CuNPs in the diet resulted in an increase ($P < 0.001$) in Cu intake by rats (Table 2). Irrespective of the level of CuNPs, the inclusion of pectin or psyllium in the diet of rats increased the level of Cu in the liver ($P = 0.009$) relative to the control group (Table 2).

Two-way ANOVA showed interactions for the amount of Cu excreted in the urine ($P = 0.006$) and faeces ($P < 0.001$), total excreted Cu ($P < 0.001$), the Cu digestibility and utilization indices ($P < 0.001$, both), and the Cu level in the muscle ($P < 0.001$) (Table 2), as well as levels of Cu ($P < 0.001$), Ca ($P < 0.001$), Mg ($P < 0.005$), Zn ($P = 0.024$) and Fe ($P < 0.001$) in the plasma of the rats (Table 3). These interactions indicate that the main effects did not significantly influence the parameters tested. The interaction observed for Cu excreted in the urine ($P = 0.006$) resulted from the fact that the combined use of inulin and the higher level of CuNPs in the diet increased Cu excretion in the urine, which was not observed in the case of the diets containing the other forms of dietary fibre and the increased level of CuNPs. The interactions observed for Cu excreted in the faeces and total Cu excreted from the body ($P < 0.001$, both) were due to the fact that the use of pectin, inulin, or psyllium in combination with the increased level of CuNPs in the diet increased excretion of Cu in the faeces and its total excretion from the body, while this effect was not observed in the group receiving cellulose together with the higher level of CuNPs. The interactions noted for the Cu digestibility and utilization indices ($P < 0.001$, both) were due to the fact that the combined inclusion of cellulose and the higher level of CuNPs in the diet increased both of these parameters in the rats, which was not observed in the groups receiving diets containing the other forms of dietary fibre together with this level of CuNPs. The interaction for the content of Cu in the muscle ($P < 0.001$) was due to the fact that the inclusion of inulin together with the increased level of CuNPs in the diet increased the level of Cu in the muscle tissue, which was not observed in the rats from the groups receiving the other forms of dietary fibre in combination with the increased level of CuNPs (Table 2). The interaction observed for the level of Cu ($P < 0.001$) in the plasma is due to the fact that the increased level of CuNPs reduces Cu content when included in the diet together with pectin, but increases it when used together with inulin, while no similar effect was observed in the groups fed diets containing the increased level of CuNPs together with cellulose or psyllium. The interaction observed for the level of Ca in the blood of rats ($P < 0.001$) was caused by the fact that it was

increased by the combined use of the increased level of CuNPs with pectin and decreased by its use with inulin, whereas similar effects were not observed in the groups receiving the other forms of dietary fibre (cellulose and psyllium) together with the increased level of CuNPs. The interactions observed for the levels of Mg ($P < 0.005$) and Zn in the blood ($P = 0.024$) were due to the fact that the combined use of cellulose and the increased level of CuNPs in the diet of rats increased the levels of these microelements, which was not observed in the case of the combined use of the other forms of dietary fibre together with the increased level of CuNPs. The interaction for the level of Fe ($P < 0.001$) in the blood of rats was due to the fact that it was increased by the use of pectin or psyllium in combination with the higher level of CuNPs in the diet, while this effect was not observed in the case of the diet containing cellulose or inulin together with this level of CuNPs (Table 3).

Discussion

The results of our research showed that neither replacing the standard form of Cu (CuCO_3) with CuNPs nor increasing their level in the diet to twice the recommended level affected diet intake or the final body weight of rats (Cholewińska et al., 2023). However, although consumption of diets containing analogous levels of CuCO_3 or CuNPs in the rats was similar, Cu intake by the rats was significantly higher in the group receiving CuNPs compared to CuCO_3 , and it increased in proportion to the increase in the level of CuNPs in the diet. Replacement of CuCO_3 with CuNPs in the diet also improved the bioavailability of Cu in the body, as indicated by the reduction in its excretion, especially in the faeces, and the increase in the digestibility and utilization indices. These results are partially consistent with the findings of our previous research, in which rats received Cu in their diets in the standard form of CuCO_3 or novel CuNPs at the recommended level of 6.5 mg/kg diet or half that level – 3.25 mg/kg diet – for four weeks. As in the present study, there was an increase in Cu intake and the utilization index, as well as a decrease in the total excretion of Cu in the urine and faeces as a result of the replacement of CuCO_3 with CuNPs (Cholewińska et al., 2018 a). These results appear to confirm reports by other authors indicating that the bioavailability of CuNPs is higher than that of their commonly used inorganic counterparts, understood as the degree to which the ingested element is absorbed from the gastrointestinal tract in a form that can then be metabolized by the body (Lee et al., 2016 a; Scott et al., 2018). There are reports that the absorption and biodistribution of CuNPs in the body most likely take place – at least to some extent – by the same mechanisms as in the case of inorganic forms of Cu. Strong oxidants present in the body can cause a portion of CuNPs to undergo oxidation to both Cu^+ and Cu^{2+} ions, particularly in the acidic environment of the stomach (Kim et al., 2008;

Ognik et al., 2016). However, the higher bioavailability of CuNPs compared to inorganic CuCO_3 is believed to be primarily due to their better solubility in the aqueous environment of the mucus coating the inside of the gastrointestinal tract, as well as to their very small size and ability to bind to proteins, which increases the range of mechanisms by which they can pass from the intestinal lumen into the body (Scott et al., 2018). It has been demonstrated that uptake of nanoparticles from the gastrointestinal tract can take place in a similar manner as in the case of ionic Cu, by active transport regulated by specific proteins forming copper channels, such as Ctr, DMT1, and ATPases 7A and 7B, or by transcytosis (mainly endo- and pinocytosis), but also by passive diffusion across mucosal cells, or even intracellular passage (Ognik et al., 2016; Scott et al., 2018). Absorption of Cu begins in the stomach, where the acidic environment causes both inorganic Cu compounds and CuNPs to dissociate into Cu^{2+} ions. Moreover, research by Lee et al. (2016 a) has shown that CuNPs dissolve rapidly in gastric juices, which additionally facilitates their absorption. Nevertheless, the predominant site of Cu absorption from the gastrointestinal contents is the small intestine, especially the duodenum, where the pH is about 6.8–7.8 (Cholewińska et al., 2018 a). Therefore the chyme that passes from the stomach to the upper parts of the small intestine is alkalized, which can result in an interaction of Cu^{2+} ions and OH^- groups, leading to the formation of insoluble copper hydroxide ($\text{Cu}(\text{OH})_2$), which does not undergo absorption. Instead, it passes through successive parts of the gastrointestinal tract and is excreted in the faeces (Lee et al., 2016 a; Scott et al., 2018; Cholewińska et al., 2018 a). Therefore it can be assumed that in the case of CuNPs, only some of them are dissociated in the stomach, while some remain in the form of neutral particles that do not undergo reactions with OH^- groups in the small intestine, which probably increases their bioavailability relative to their inorganic macro counterparts.

After being absorbed in the small intestine, Cu enters the liver through the portal vein. As much as 75% of the Cu from the intestines is retained in this organ, while only 25% of Cu, bound with proteins (mainly albumins), flows directly to the peripheral blood. About 80% of the copper retained in the liver is bound to ceruloplasmin synthesized in the hepatocytes, and in this complex, it is released into the peripheral blood, from which it reaches the other organs. The remaining 20% of Cu is bound to bile produced in the liver, forming insoluble complexes which are then released into the intestinal lumen and excreted from the body in the faeces (Ognik et al., 2016; Cholewińska et al., 2018 a). The literature indicates that the binding of Cu to bile is a defence mechanism against the toxic effects of excessive Cu and is the predominant route of Cu excretion from the body (Wapnir, 1998; Gupta and Lutsenko, 2009; Bost et al., 2016; Scott et al., 2018; Cholewińska et al., 2018 a, b). While a large amount of Cu also passes into primary urine synthesized in the kidneys, it is resorbed back into the blood in the proximal re-

nal tubules, from which only 2% of Cu is excreted in the urine (Wijmenga and Klomp, 2004). Lee et al. (2016 a) compared the excretion of various forms of Cu from rats and found that ionic Cu was excreted in the urine to a greater extent than CuNPs, which were excreted mainly in the faeces. Our results showed that replacing CuCO_3 with CuNPs in the diet of rats reduces the excretion of Cu from the body, especially in the faeces, enabling its better utilization in the body, as indicated by the increase in the digestibility and utilization indices.

The literature indicates that an increase in the plasma level of Cu can be a good marker of intestinal absorption. Interestingly, the results of our study showed that the replacement of CuCO_3 with CuNPs in the diet not only did not increase the level of Cu in the blood of rats but even decreased it. Moreover, Lee et al. (2016 a) reported that Cu administered to rats in the form of inorganic compounds such as carbonate or sulphate accumulated mainly in the liver, brain, and kidneys, whereas CuNPs additionally accumulated in the spleen, lungs and heart (Lee et al., 2016 a, b). The results of our previous research (Cholewińska et al., 2018 b) also showed a tendency of CuNPs to penetrate the blood–brain barrier and accumulate in the nervous tissue, whereas they were not found to accumulate in the liver, even in the case of the higher level in the diet (6.5 mg/kg diet). Therefore it is particularly interesting that in the present study not only did replacement of CuCO_3 with CuNPs not result in excessive accumulation of Cu in the liver, but it reduced the Cu level in the blood, brain and muscle. These findings are somewhat surprising and difficult to explain. Nevertheless, in view of the other results obtained in the experiment, such as the increase in Cu intake accompanied by a decrease in its excretion and the beneficial effect on its digestibility and utilization, the overall effect should be regarded as favourable. The reduction in the Cu level in the blood can be assumed to be due to the need for large amounts of this mineral and its involvement in metabolic processes.

The literature indicates that the level of absorption of microelements, including Cu, can be significantly modified not only by their form but also by other nutrients present in the diet, including dietary fibre (Gralak et al., 1996; Kim and Shin, 1996; El-Zoghbi and Sitohy, 2001; Coudray et al., 2006; Krzysik et al., 2009). Although intake of dietary fibre has significant health benefits, e.g. by helping to maintain normal body weight, improving the glycaemic response, and reducing the risk of cardiovascular disease and colorectal and breast cancer (Maćkowiak et al., 2016), it can also inhibit intestinal absorption of trace elements (Rockway et al., 1987; Caballero, 1988; Adams et al., 2018). This may be due to the physicochemical properties of dietary fibre, such as the ability to ferment, dissolve, bind water, form a gel, swell, or bind cations – especially divalent cations (Krzysik et al., 2009; Baye et al., 2017; Capuano, 2017). Moreover, dietary fibre increases peristalsis, reducing the time that food is in contact with the enterocytes, and enhances acid

secretion and release of hormones, which can also affect absorption processes (Caballero, 1988; Spiller, 2001; Krzysik et al., 2009). However, literature data on the effect of dietary fibre on the absorption of elements from the digestive tract are often highly inconsistent. Krzysik et al. (2009) observed a decrease in the absorption of divalent ions, including Cu ions, in rats receiving a diet containing pectin or cellulose. Drews et al. (1979) and Turnlund et al. (1985) did not confirm a negative effect of these forms of dietary fibre on Cu absorption processes in young boys, while Coudray et al. (2006) additionally reported increased absorption and retention of Cu in the intestines following intake of inulin. The results of our study also showed an increase in the Cu level in the liver of rats that received a diet containing pectin or psyllium. Copper absorbed in the small intestine reaches this organ first and may be metabolized in it, and even accumulate. The increase in the level of Cu observed in the liver of rats receiving a diet with pectin or psyllium, irrespective of the level of CuNPs in the diet, therefore suggests that they not only do not inhibit processes of intestinal absorption of Cu, but even stimulate them.

According to Coudray et al. (2006), absorption of minerals from the digestive tract may depend not only on the amount and type of dietary fibre, but also on the homeostasis of minerals in the body. This seems to be supported by the results of our study, which indicate the occurrence of a number of interactions between CuNPs and dietary fibre, but only in the case of the higher level of CuNPs in the diet. The combined use of pectin, inulin, or psyllium with the higher level of CuNPs in the diet of rats increased the excretion of Cu in the faeces, and in the case of inulin in the urine as well. There are reports that binding of Cu to bile and its secretion into the digestive tract plays the most important role in controlling the homeostasis of this microelement in the body (Harvey et al., 2003; Bost et al., 2016). Therefore it is likely that when Cu intake in the diet is too high, the body increases the rate of processes of its excretion to protect against potential toxic effects and accumulation in certain organs. This seems to be supported by our previous research, which also showed a dose-dependent increase in Cu excretion in rats fed a diet containing CuNPs. In the present study, the effect was additionally enhanced by the forms of dietary fibre tested – pectin, inulin and psyllium. This may be due to the fact that these three forms are examples of soluble dietary fibre, which increases its bulk by binding water. This results in a viscous gel (especially in the case of pectin and psyllium) capable of binding divalent ions such as Cu^{2+} to free hydroxyl groups. Therefore excessive Cu in the chyme in contact with dietary fibre was incorporated into insoluble complexes unavailable for intestinal absorption processes, which were then excreted in the faeces (El-Zoghbi and Sitohy, 2001; Asvarujanon et al., 2004; Krzysik et al., 2009). This suggests that the higher the level of CuNPs in the diet, the more effectively the alternative forms of dietary fibre – pectin, inulin and psyllium – protect the body against their potentially

harmful impact by increasing their excretion from the body. This may also be confirmed by the fact that despite the tendency of CuNPs to accumulate in internal organs such as the liver or brain, observed in our earlier study and by other authors, the addition of alternative forms of dietary fibre to the diet effectively prevented this process even when dietary intake of CuNPs was twice the recommended level for rats. Moreover, the inclusion of inulin in the diet of rats additionally reduced the level of Cu in the muscle. Among the alternative sources of dietary fibre tested in the study, inulin in the diet of rats caused the greatest increase in excretion of Cu when intake of CuNPs exceeded the recommended level, which most likely translated to a reduction in the Cu level in the body and thus to lower accumulation in the tissues. This is because Cu is first utilized for copper-dependent physiological processes such as the synthesis of antioxidant enzymes or enzymes involved in cellular respiration. This assumption also seems to be supported by the increase in the Cu level noted in the blood as a consequence of the administration of inulin together with the higher level of CuNPs in the diet of rats. Cu is mobilized in the blood and together with it reaches its target cells, in which it is then incorporated into copper-dependent metabolic pathways. The reduction in the Cu level in the muscle and the lack of this effect in the liver or brain as a result of the combined use of inulin and the higher level of CuNPs additionally indicates that among the tissues analysed, the muscle shows the least tendency to store Cu. This is in line with the findings of other authors reporting that CuNPs show the highest affinity for parenchymal organs such as the liver, kidneys, spleen (Lee et al., 2016 a), or brain (Cholewińska et al., 2018 b).

Cellulose, on the other hand, is an example of an insoluble form of dietary fibre, and thus it does not have a clear tendency to bind water (McRorie and McKeown, 2017). In the present study, the combined use of cellulose and the increased level of CuNPs increased the digestibility and utilization of copper in the rats. The literature indicates that the digestibility of minerals reflects processes of dissolution and absorption of nutrients from the intestinal lumen. It has also been demonstrated that Cu digestibility can be significantly increased by a Cu deficiency, such as one induced by inhibition of its absorption. The body then attempts to maintain Cu homeostasis and activates processes of increased synthesis of Cu transport proteins, and the Cu-ATPase pump is used to transport Cu through the basolateral membrane to the extracellular fluid (Espinosa and Stein, 2021). The increase observed in the digestibility of Cu in rats in the present study may therefore suggest that in the case of increased intake of CuNPs in the diet, cellulose significantly restricts its absorption. Nevertheless, due to the lack of increased excretion of Cu from the body or deterioration of other significant parameters indicative of Cu homeostasis in the body, this assumption cannot be confirmed. It even appears that during increased intake of CuNPs, cellulose, in contrast to pectin, inulin or psyll-

ium, does not limit Cu absorption at all, or does so to a lesser extent, and improves their retention and utilization. This is in line with the findings of Drews et al. (1979) and Turnlund et al. (1985), who also found no deterioration of Cu absorption from the gastrointestinal tract of young boys receiving a cellulose-rich diet. In the case of a high level of Cu in the diet, especially in the form of CuNPs with potentially better bioavailability, this effect may not be entirely favourable, as it may be associated with excessive accumulation of CuNPs in the body, leading to the manifestation of their toxic effects (Cholewińska et al., 2018 a, b). However, our study did not show that the combined use of cellulose and an increased level of CuNPs in the diet of rats resulted in excessive accumulation of copper in the internal organs.

The bioavailability of minerals is well known to be influenced by antagonistic interactions as well, in particular between Cu, Zn, Fe, and Ca. The results of our previous research suggest that the occurrence of these interactions stems mainly from the fact that all of these elements form divalent cations, which can be absorbed from the digestive tract by the same transport mechanisms (Ognik et al., 2016). This increases their competition, e.g. for binding to transport proteins Ctr1 and DMT1 (Ognik et al., 2016). When the diet is well-balanced, unfavourable changes in the bioavailability of these microelements are generally not observed, because their levels are not high enough to induce antagonistic interactions resulting from excessive amounts of elements relative to the levels of transport proteins (Gibson, 2007). Moreover, micronutrients in the digestive tract become chelated with dietary ligands as a result of the digestion of food and in this way are absorbed by various routes (Sandström et al., 1985; Gibson, 2007). However, interactions can become problematic when one microelement is included in the diet in excess relative to the others (Gambling et al., 2011). For example, excessive zinc in the diet (25 or 50 mg/day) has been shown to reduce biochemical indicators of the level of copper in adults (Fischer et al., 1984; Yadrick et al., 1989) and in some cases iron as well (Yadrick et al., 1989). Potential interactions have also been shown to be influenced by the form in which a microelement is ingested with the diet (Gheisari et al., 2011). The results of our study indicate that the replacement of CuCO_3 with CuNPs in the diet of rats reduced the Cu level in the blood while increasing the levels of other trace elements present in the form of divalent cations, such as Ca, P, Mg, Zn, and Fe. The results are somewhat surprising, and in light of the above it could be assumed that they are explained by decreased intestinal absorption of Cu, enabling better absorption of the other minerals analysed, and thus higher levels of these elements in the blood serum. However, this is not confirmed by the other findings of our study, such as the increase in the intake of CuNPs and their retention and utilization in the body. Therefore it can be postulated that CuNPs themselves are very well absorbed from the gut without negatively affecting the absorption of other microelements. This may be explained by the fact that at

least a certain pool of the CuNPs present in the ingesta occur in the form of neutral particles which can avoid antagonistic interactions with other microelements. It is likely that CuNPs can additionally be absorbed by alternative transport mechanisms, such as paracellular transport, which is possible owing to their very small size (Ognik et al., 2016). This means that CuNPs most likely use Ctr1 and DMT1 for transport purposes to a lesser extent, leaving the largest portion of them at the disposal of other divalent cations. This translates to their better intestinal absorption and higher levels in the blood serum and makes it possible to meet the demand for all micro-nutrients needed for metabolic processes.

The present study also showed that the addition of dietary fibre significantly influenced the content of Cu and the other minerals tested in the blood, but only when an excessive level of CuNPs was administered to the rats. The inclusion of pectin in the diet with a higher level of CuNPs resulted in a decrease in the Cu level and an increase in the Fe level in the blood of rats. An increased plasma level of Fe was also noted in rats receiving psyllium together with the increased level of CuNPs. Maintaining an adequate level of Fe in the body is extremely important because iron takes part in oxygen storage and transport as a component of haemoglobin, as well as supporting detoxification processes, immune defences, and prostaglandin production (Abbaspour et al., 2014). The literature indicates that elevated levels of Cu competitively inhibit iron absorption and utilization and are positively correlated with the decrease in iron in the serum (Hébert et al., 1993; Arredondo and Núñez, 2005; Lee et al., 2016 a). The increased plasma Fe level observed in the present study accompanied by increased excretion of Cu in the rats receiving a high level of CuNPs in their diet therefore suggests that pectin, inulin, and psyllium all protect the body against excessive absorption of Cu, thereby maintaining its homeostasis. Our results indicate that in the case of excessive intake of CuNPs, cellulose can also significantly modify the interactions between Cu and other microelements, such as Zn or Mg. Magnesium ranks fourth in quantity among elements present in living organisms. It is involved in the regulation of skeletal and cardiac muscle contractility, remodelling and mineralization of bone tissue, and transmission of impulses in the nervous system. Moreover, as a cofactor of numerous enzymes it takes part in metabolism of proteins, carbohydrates, lipids and nucleic acids (Glasdam et al., 2016; Al Alawi et al., 2018). Like magnesium, zinc is also involved in the synthesis of many enzymes, e.g. those ensuring biosynthesis of nucleic acids and protein and antioxidant defence. It is also essential to normal development and cell division, influences the synthesis and function of steroidal hormones, and takes part in regulation of energy processes in the body (Roohani et al., 2013). Given the importance of these elements for the functioning of the body, it is essential to ensure that their levels are adequate. The results of our study showed that the addition of cellulose to a diet for rats containing

a high level of CuNPs increased levels of Mg and Zn in the blood without negatively affecting the level of Cu. This suggests that the presence of cellulose in the diet, like that of pectin or psyllium, can beneficially influence the interrelationships between trace elements in the case of excessive intake of Cu.

To sum up, the replacement of CuCO_3 with CuNPs in the diet of rats beneficially influenced the biodistribution of Cu in the body by reducing its excretion – especially in the faeces, improving its digestibility and utilization, reducing its accumulation in the brain and muscle, and increasing levels of Ca, P, Mg, Zn and Fe in the blood. As expected, increasing the level of CuNPs in the diet from 6.5 mg/kg to 13 mg/kg increased the total intake of Cu. All three alternative forms of dietary fibre – pectin, inulin and psyllium – added to the diet with high content of CuNPs significantly increased excretion of Cu, with no negative effect on its digestibility and utilization, and prevented its excessive accumulation in the brain and muscle of rats, especially in the case of inulin. This effect may be regarded as a form of protection against potentially harmful excessive amounts of Cu. The addition of pectin and psyllium to a diet containing a high level of CuNPs additionally increased the level of Fe in the blood of rats. The results suggest that the addition of dietary fibre to the diet of rats ensures homeostasis of this element in the case of excessive intake of CuNPs by modifying the bioavailability of Cu. In this way, it effectively protects the body against their potential harmful effects, which can translate to beneficial regulation of their metabolic effect on the body.

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