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HIPPOCAMPUS AND JEJUNUM BIOCHEMICAL PARAMETERS RELATED TO PHYSIOLOGICAL AGEING OF NEURONS IN RATS FED DIETS WITH COPPER NANOPARTICLES AND DIFFERENT FIBER TYPES*

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Abstract

One of the more important factors responsible for synaptic dysfunction of neurons is neurological ageing. It is a natural consequence of occurrence of biochemical pathways in cells, promoting oxidation processes, an increase in the amount of free oxygen radicals that damage DNA, disorders in calcium ion homeostasis, interactions between lipids of cell membranes, formation of neurofibrillary tangles and deposition of toxic protein deposits. Diet modifications can delay neurometabolic disorders that occur as a result of ageing, as well as limit the development of neurodegenerative diseases. The aim of this study on rats was to verify the hypothesis regarding the effect of dietary addition of copper nanoparticles (CuNPs; instead of standard $CuCO_3$) in combination with various dietary fibers on changes in hippocampus and jejunum biochemical parameters related to metabolic disorders appearing with neurological ageing in rats. Male rats were fed a control diet with fiber α-cellulose and a standard or enhanced content of Cu provided as $CuCO_3$ (6.5 or 13 mg/kg diet). The diets of other groups were deprived of $CuCO_3$ and the animals were fed diets supplemented with two CuNPs aforementioned doses, and additionally combined with four different types of dietary fiber: α-cellulose (control fiber), pectin, inulin or psyllium (*Plantago psyllium*). The addition of dietary CuNPs increased the level of acetylcholinesterase (AChE) and decreased the levels of Tau protein, glycosylated AChE (GAChE) and Ca/calmodulin dependent protein kinase II alpha (CAMK2α) in the examined organs. To sum up, both applied doses of CuNPs, 6.5 mg and 13 mg per kg of a diet, can beneficially modulate some important parameters describing metabolic and neurodevelopmental status in the hippocampal and small intestinal tissue of rats. With regard to the fiber type added to rats' diet, the *Plantago psyllium* fiber strengthened the mentioned effects of copper nanoparticles. The observed effects should be confirmed in a model study of neurodegeneration.

Key words: copper nanoparticles, copper carbonate, dietary fiber, hippocampus, jejunum, metabolic and neurodevelopmental status

Ageing of the animal nervous system is a dynamic process accompanied by changes in physiological and biochemical functions and a progressive degradation of anatomical structures. The changes in neurometabolism are a natural consequence of occurrence of biochemical pathways in cells, promoting oxidation processes, an increase in the amount of free oxygen radicals that damage DNA, disorders in calcium ion homeostasis, interactions between lipids of cell membranes, formation of neurofibrillary tangles and deposition of toxic protein deposits. We can observe the spectrum of these changes at the level of biomolecules, nerve cells and finally entire brain regions (Kawahara et al., 2011; Wahl et al., 2016; Wu and Hsieh, 2023). The consequences of changes in neurometabolism therefore may be neurodegenerative diseases, which are associated with progressive neuronal damage and ultimately death of nervous cells. Diet modifications can delay the neurological ageing process by slowing down the development of age-related neurometabolic disorders and limiting the development of neurodegenerative diseases (Flanagan et al., 2020; Gallo et al., 2022). Despite the growing number of studies on this subject, literature data are still insufficient, they raise many doubts and further questions. Therefore, dietary fiber may be an interesting subject for research into Alzheimer's disease (Taylor et al., 2017; Stefaniak et al., 2022). According to Shi et al. (2021) dietary fiber deficiency may impair cognitive function. De Leeuw et al. (2008) believes that it also positively modifies behavioural responses. Animals consuming the ration of feed, which meets the need for high-quality dietary fiber, are definitely calmer, do not show symptoms of stereotypic self-directed behavior, are less physically active and do not show agitation associated with searching for food. This reaction results from

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the high potential of prolonging the feeling of post-meal satiety and proper motivation for many hours after eating (De Leeuw et al., 2008). Fiber also reduces the risk of type II diabetes, which is considered one of the risk factors for the development of Alzheimer's disease (Stefaniak et al., 2022, Crane et al., 2013; Kimura et al., 2021).

Copper is involved in the regulation of many biochemical processes, immunological response, Fe metabolism, the synthesis of neurotransmitters, neuropeptides, hormones and regulation of the metabolism of the nervous tissue, and neuron myelinization. It is a co-factor for many enzymes, especially those that are essential for maintaining metabolism and production of energy as well as antioxidant response (Calvo-Rodriguez et al., 2020; Cendrowska-Pinkosz et al., 2022; Cholewińska et al., 2018 a, b). Covering the brain's demand for energy requires an adequate supply of ATP. The energy material for neurons is glucose, which is oxidized through glycolysis and oxidative phosphorylation to produce ATP (Ashrafi and Ryan, 2017). A large amount of energy is necessary to ensure the maintenance of the ion gradient and restore the proper concentration of Ca^{2+} in mitochondrion. ATP deficiencies can occur when too much Ca^{2+} flows into the mitochondrion, for example when B-amyloid levels increase, as demonstrated by Calvo-Rodriguez et al. (2020) in a mouse model. Copper is a catalytic cofactor for enzymes that affect energy metabolism (cytochrome C oxidase) and crucial for redox balance and antioxidant defence (SOD1 and SOD2) (Tapiero et al., 2003; Angelova et al., 2011; Blomberg, 2021). In neurometabolic diseases (i.e., Wilson and Menkes disease), copper accumulates in the liver due to a malfunctioning ATP7B protein transporter. Copper overload impairs cellular respiration, increases free radical production and induces apoptosis (Ruiz et al., 2021). However, copper deficiency can also lead to cellular respiration dysfunction, deregulation of the metabolism as well as immune system defects (Cendrowska-Pinkosz et al., 2022). The overabundance of copper can stimulate the overproduction of free radicals. On the other hand, copper deficiency is related to the disruption of cellular respiration as well as metabolic and immunological deregulation. Due to the high reactivity of inorganic copper sources (carbonates, sulfates, chlorides), which are usually supplemented in the diet, and the ability of copper to interact with other nutrients (via oxidation), it is currently important to search for different sources of copper that are better absorbable via the digestive tract (Cendrowska-Pinkosz et al., 2022; Cholewińska et al., 2018 a, b). Copper nanoparticles (CuNPs), which are unique by their small sizes and easier bioavailability (Cholewińska et al., 2018 a) can satisfy this demand. Those nanoparticles are characterized by a larger active surface and the availability of deeper penetrance of the intestinal mucosa. It is believed that CuNPs might induce oxidative stress, inflammation, and DNA damage or inhibit cell proliferation (Cholewińska et al., 2018 b). Moreover, the excess amount of copper might have a damaging effect on the nervous system (Cendrowska-Pinkosz et al., 2022). This information, therefore, raises concerns about the expanding usage of CuNPs in animal production and their possible influence on neurometabolic damage due to the deposition of copper in various tissues. Metabolic and neurodevelopmental disorders alter the structure and physiology of the hippocampus (Li et al., 2019). These changes are the result of the coexistence of mitochondrial dysfunction (disrupted cellular energetics), the damage of oxidative biomolecules, neural inflammation, and deregulation of apoptotic cell death (Angelopoulou et al., 2021; Wang et al., 2019). Additionally, a vital factor in the correct function of the brain is the close relationship between the central nervous system (CNS) and the intestinal nervous system (INS). It is maintained by the neural connections between the tissues, metabolic processes, and the existing intestinal immune response. Moreover, there is close communication between the intestinal microbiota and the nervous system, provided by the CNS and INS (Carabotti et al., 2015).

Current scientific findings provide information on the role of dietary fiber as potential prevention of neurodegeneration changes (Stefaniak et al., 2022). Dietary fiber has been found to enhance the development of the intestinal microbiota, decrease the formation of free radicals as well as modulate the amount of neurotrophic brain-derived factors, which are responsible for the development and function of the nervous system (Divyashri et al., 2021; Fernando et al., 2018; Yamagishi et al., 2023). Alzheimer's disease is associated with progressive damage to the cells that build structures belonging to the nervous system. The main histological features of neurodegeneration are senile plaques, neurofibrillary tangles, and extensive neuronal death. Outside the neurons and in the cerebral vessels, there are the so-called senile plaques, which consist mainly of proteins and peptides, often glycosylated, called β-amyloid. At the same time, oligomers of abnormally phosphorylated Tau protein of the cytoskeleton accumulate inside the neurons (Tönnies et al., 2017; Götz et al., 2004). Tau protein is a physiological compound of the neurons. The potential neurotoxic properties of Cu and CuNPs, which disrupt neural metabolism, might result in the formation of other toxic compounds. Those compounds are likely to cause mitochondrial dysfunction and the formation of reactive oxygen species (ROS) and induce neural apoptosis. Such compounds are glycosylated acetylcholinesterase (GAChE) (Sáez-Valero et al., 2003), which is a complex of the enzyme acetylcholinesterase with βAP and Tau protein, and hyperphosphorylated Tau protein (p-Tau), formed as a result of phosphorylation of Tau protein under the influence of the complex of calmodulin and Ca^{2+} (CAMK2a). Consequently, p-Tau disrupts the functioning of synaptic mitochondria (Tönnies et al., 2017). Notably, impaired functioning of neural tissue is observed during a decreased level of acetylcholinesterase (AChE) as well as during excessive synthesis of this enzyme, which determines the importance of AChE as the key enzyme for

the correct neural conduction and neuroprotection. In addition, low-density lipoprotein receptor-related protein 1 (LRP1), is another important enzyme that helps degrade βAP (S**á**ez-Valero et al., 2003). Lipids are the essential compound of brain tissue, therefore lipid transporters such as ATP-binding cassette transporters (ABC) are crucial to maintaining proper functioning and homeostasis of the CNS. The ABC system is a group of highly differentiated, membrane-bound transporting proteins, which participate in the process of intracellular ATP hydrolysis and enable intermembrane transport of nutrients (Blomberg, 2021). Furthermore, neurometabolism is regulated by the activity of calmodulin and Ca^{2+} complex. This complex activates protein kinases, which phosphorylate proteins contributing to the synthesis of toxic p-Tau. The disturbed calcium homeostasis combined with the damaged neuronal cell membrane integrity leads to neuronal death (Rowe et al., 2019). An indicator of epigenetic changes is cytosine methylation in DNA (Cendrowska-Pinkosz et al., 2021).

It was assumed that exchanging traditionally administered, carbonated form of copper ($CuCO_3$) for copper nanoparticles (CuNPs) and combining CuNPs with different kinds of dietary fiber may have a substantial effect (positive or negative) on the physiological ageing of central nervous system (i.e., hippocampus) and neurons of peripheral tissues (in the case of this study, the jejunum tissue) with regards to changes of chosen biochemical parameters. In the case of the negative effect of copper nanoparticles, we hypothesize that such an effect may be mitigated by the presence of dietary fiber in the diet.

Material and methods

Animal protocol and dietary treatments

In vivo experiment was performed using healthy outbred 9-week-old male Wistar rats (Cmdb:Wi CMDB). All animal care and experimental protocols were compiled according to 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 Local Ethics Committee for Animal Experiments (Approval No. 19/2021). The experimental factor were different sources of copper (Cu carbonate – $CuCO_3$ and nanoparticles – CuNPs) and different levels of Cu in the diet. The copper content of the diet samples was determined by inductively coupled plasma emission spectrometry (ICP-OES, Varian Inc., Palo Alto, CA, USA). In the experiment all nanoparticle diets were modified by adding different sources of fiber: cellulose (control), pectin (with viscous effect), inulin (with fiber prebiotic effect) and *Plantago psyllium* (with bulk effect). The used diets were modifications of a standard casein diet for rats, recommended by the American Institute of Nutrition (AIN) (Table 1).

Table 1. Composition of diets used in the experiment on rats, %

Diet	Control C Control CH	CN CNH	PN PNH	JN JNH	SN SNH
Casein	14.8	14.8	14.8	14.8	14.8
DL-methionine	0.2	0.2	0.2	0.2	0.2
Cellulose	8.0	8.0	2.0	2.0	2.0
Pectin	–	–	6.0	–	–
Inulin	–	–	–	6.0	–
Plantago psyllium	–	–	–	–	6.0
Choline chloride	0.2	0.2	0.2	0.2	0.2
Rapeseed oil	8.0	8.0	8.0	8.0	8.0
Cholesterol	0.3	0.3	0.3	0.3	0.3
Vitamin mix[a]	1.0	1.0	1.0	1.0	1.0
Mineral mix[b]	3.5	3.5	3.5	3.5	3.5
Maize starch	64.0	64.0	64.0	64.0	64.0
	Calculated dietary additional Cu level from two sources, mg/kg of a diet				
$CuCO_3$	6.5 (C diet) 13 (CH diet)	–	–	–	–
CuNPs	–	6.5 (CN diet) 13 (CNH diet)	6.5 (PN diet) 13 (PNH diet)	6.5 (JN diet) 13 (JNH diet)	6.5 (SN diet) 13 (SNH diet)

Control groups C and CH, fed a diet with standard and enhanced Cu content (6.5 and 13 mg/kg from $CuCO_3$, respectively). In the remaining groups, the diets were supplemented with copper nanoparticles (CuNPs) at two doses: 6.5 mg/kg (groups CN, PN, JN, SN) or 13 mg/kg (groups CNH, PNH, JNH, SNH). The dietary fiber in the diets: 8% of cellulose (groups C, CH, CN, CNH), 6% of pectin + 2% of cellulose (groups PN, PNH), 6% inulin + 2% cellulose (JN, JNH), 6% psyllium + 2% cellulose (SN, SNH).

[a]AIN-93G-VitaminMixture.

[b]Changeable dietary ingredient in relation to copper level; mineral mixture with different Cu level originated from two sources (standard source $CuCO_3$ and experimental CuNPs preparation).

Table 2. Experimental details with regard to dietary fiber and additional copper applied to rats for 6 weeks (provided Cu dose was calculated taking into account $CuCO_3$ in mineral mixture or copper from Cu nanoparticles preparation, CuNPs)

Treatment	Source of additional Cu and dietary fiber
Control C	6.5 mg/kg Cu of additional dietary Cu from $CuCO_3$ and 8% of cellulose (n=8)
Control CH	13 mg/kg Cu of additional dietary Cu from $CuCO_3$ and 8% of cellulose (n=8)
CN	6.5 mg/kg of additional dietary Cu from CuNPs and 8% of cellulose (n=8)
CNH	13 mg/kg of additional dietary Cu from CuNPs and 8% of cellulose (n=8)
PN	6.5 mg/kg of additional dietary Cu from CuNPs and 2% of cellulose + 6% of pectin (n=8)
PNH	13 mg/kg of additional dietary Cu from CuNPs and 2% of cellulose + 6% of pectin (n=8)
JN	6.5 mg/kg of additional dietary Cu from CuNPs and 2% of cellulose + 6% of inulin (n=8)
JNH	13 mg/kg of additional dietary CuNPs and 2% of cellulose + 6% of inulin (n=8)
SN	6.5 mg/kg of additional dietary Cu from CuNPs and 2% of cellulose + 6% of *Plantago psyllium* (n=8)
SNH	13 mg/kg of additional dietary Cu from CuNPs and 2% of cellulose + 6% of *Plantago psyllium* (n=8)

= 8, number of rats used in experimental feeding period.

The rats aged 9 weeks were randomly divided into 10 groups with n = 8 per one group (Table 2). Before the start point, for 5 preliminary days all rats were fed the standard casein-based diet (containing 6.5 mg/kg of dietary Cu from $CuCO_3$ which is a standard component in mineral mixture). The feeding experiment lasted 6 weeks. The rats were kept individually in cages and facilities that provided suitable comfortable conditions for this type of animal research. Experimental diets were supplemented with a standard dose of additional Cu (6.5 mg/kg of diet; the same diet as in the preliminary 5-day period; a typical control diet used in nutritional studies on rats) and two times higher dose of additional Cu (13 mg/kg of diet), as commonly used in rodent diets ($CuCO_3$) or novel source as nanoparticles (CuNPs), or both. The experimental diets also differed in terms of dietary fiber types, applied in the form of pectin, inulin, or *Plantago psyllium*, which were added at 6% of a diet at the expense of the same amount of cellulose which was considered as a control fiber type (Table 2).

The following groups were created: with standard and double dose of additional Cu originated from $CuCO_3$, a standard ingredient of common mineral mixture for laboratory rodents (6.5 and 13 mg/kg of diet, respectively; groups: C and CH), and two corresponding levels of CuNPs (6.5 and 13 mg/kg of diet; in that case the mineral mixture was deprived of $CuCO_3$). Additionally to copper nanoparticles (CuNPs) content, the diets were supplemented with different types of dietary fiber: cellulose (diets CN and CNH with lower and higher CuNPs addition, respectively), pectin (diets PN and PNH with lower and higher CuNPs addition, respectively), inulin (JN and JNH with lower and higher CuNPs addition, respectively) and *Plantago psyllium* (diets SN and SNH with lower and higher CuNPs addition, respectively). In order to keep the operator safe while preparing the experimental diets, the CuNPs preparation was added to a diet as an emulsion along with dietary rapeseed oil.

Characterization of the sources of Cu

Nanoparticles characterization: copper nanoparticles were obtained from Sky Spring Nanomaterials Inc. (Houston, TX, USA), with purity of 99.9%, 40–60 nm in size (nanopowder), spherical morphology, 0.19 g/cm^3 bulk density, and 8.9 g/cm^3 true density. The zeta potential of CuNPs was determined to be 30.3 mV (phosphate-buffered saline), Cu carbonate (purity ≥99 %) was sourced from POCh (Gliwice, Poland).

Experimental procedures

The rats were housed randomly and individually in stainless steel cages under a stable temperature (22±1°C), relative humidity 60±5%, a 12 h light-dark cycle, and a ventilation rate of 15 air changes per hour. The rats were carefully observed by trained technical staff to recognize any indicators of animal's fear, distress, pain or anxiety. For 6 weeks, the rats were given free access water and diets, which were prepared and then stored at 4°C in hermetic containers until the end of the experiment. All measurements were made separately for each animal (n = 10 for each group). The rats were fasted for 12 hours and were anesthetized by intraperitoneal injection of ketamine + xylazine (100 mg/kg + 10 mg/kg of body weight) according to recommendations for anesthesia and euthanasia of experimental animals. The rats were weighed and finally, they were euthanized by cervical dislocation and after that the hippocampus and jejunum were collected from animals. Homogenates from the hippocampus and jejunum were prepared in accordance with the analytical procedure recommended by the manufacturer of the ELISA enzyme immunoassay, by means of which selected indicators in the tissues were determined. For this purpose, 1 g of hippocampus or jejunum tissue was weighed and homogenized in ice with 9 ml PBS (0.02 mol l, pH 7.2) using a porcelain knife. The resulting mixture was then centrifuged for 15 min at 1500 × g. After removing the supernatant, the samples were stored at –80°C for further analyses.

Laboratory analysis

All analyzed indicators were determined in homogenates of brain (hippocampus) and jejunum tissues. Each sample was analyzed in 6 replicates. Selected indicators were determined using commercial ELISA kits (Sigma-Aldrich, St. Louis, MO, USA). An RT6900 microplate reader (DRG MedTek) was used to read absorbance by the immunoenzymatic method (double binding test – sandwich ELISA). The level of acetylcholinesterase (AChE) was determined by using Rat Acetylcholinesterase ELISA Kit (Bioassay Technology Laboratory, Inc., China) and the level of amyloid-β using Rat Total β-amyloid Protein (βAP) ELISA Kit (Blue Gene Biotech, Shanghai, China). The level of glycosylated acetylcholinesterase (GACHE) was determined using Rat Glycosylated Acetylcholinesterase ELISA Kit (Blue Gene Biotech, Shanghai, China). Tau protein was determined by using rat Tau Protein (Tau) ELISA Kit, and the level of phosphorylated p-Tau protein was determined using the Rat Phosphorylated tau 231 (p Tau 231) ELISA (Blue Gene Biotech, Shanghai, China). Low-density lipoprotein receptor-related protein 1 (LRP1) was determined using a Rat Low Density Lipoprotein Receptor Related Protein 1 ELISA Kit (Blue Gene Biotech, Shanghai, China). ATP-binding cassette transporters were determined by Rat ATP Binding Cassette Transporter G2 ELISA Kit (ABC), and kinase II with using rat Calcium/Calmodulin Dependent Protein Kinase II Alpha (CAMK2a) ELISA kit (Blue Gene Biotech, Shanghai, China). The levels of epigenetic changes in the hippocampus and jejunum were determined by analyzing global DNA methylation (% mC) using diagnostic kits from Sigma Aldrich.

Statistical analysis

The data obtained from the groups fed diets with CuNPs were subjected to 2-way ANOVA to examine the main effects: F – effect source of fiber (4 types of fiber: cellulose, pectin, inulin or *Plantago psyllium*) and D – CuNPs dietary dose effect (6.5 and 13 mg CuNPs, per one kg of a diet). If the analysis revealed a significant interaction ($P \leq 0.05$), the differences among the respective treatment groups (CN, CNH, PN, PNH, JN, JNH, SN SNH) were then determined with the Newman-Keuls post hoc test at $P \leq 0.05$. The statistical analysis was performed according to the GLM procedure for Statistica 10.0 software (StatSoft Corp., Kraków, Poland). The model assumptions of normality and homogeneity of variance were examined by the Shapiro-Wilk and Levene tests, respectively. Additionally, each aforementioned group fed a diet with 6.5 mg/kg CuNPs (CN, PN, JN, SN) was compared to the control C group (6.5 mg/kg of additional Cu from $CuCO_3$) with the aid of two-tailed Dunnett's post hoc-test. Similar comparisons were made with the CNH, PNH, JNH, SNH groups (13 mg/kg CuNPs) and the CH group (13 mg/kg of additional Cu from $CuCO_3$). Treatment effects were considered to be significant at $P \leq 0.05$. All data were expressed as mean values with pooled SEM (standard error of the mean). SEM was calculated by using SD for all rats divided by square root of rat number (n = 100).

Results

The level of analyzed indicators in the hippocampus

The animals did not show any negative behavioral signs related to the experimental factors, i.e., the content of copper nanoparticles and the presence of different types of dietary fiber. The Dunnett's test showed that the addition of CuNPs significantly increased the level of AChE and decreased the levels of Tau protein, GAChE and CAMK2a in hippocampus compared with control groups (C and CH) which received inorganic copper in the form of $CuCO_3$ (Table 3; Figures 1 and 2).

Regardless of the dietary fiber type, the addition of 6.5 mg of CuNPs increased the level of LRP1 in comparison with the C group. Similarly, in the group which received 13 mg of CuNPs there was an increase in LRP1 level compared with the CH group, and the addition of fiber did not differentiate the two groups according to this parameter. A comparison with the CH group showed an increased percentage of DNA methylation in the hippocampus of the animals which received 13 mg of CuNPs. However, for the dosage of 6.5 mg of CuNPs increased DNA methylation was observed among the CN and PN groups. A comparison with the control group which received inorganic copper demonstrated a significant decrease of p-Tau levels in the hippocampus of all subjects except for the rats from the JN group. Increased level of ABC was observed in the PNH, JNH and SNH groups compared with the control CH group. Two-way ANOVA analysis of the experiment performed on the rat hippocampus showed that applying different kinds of dietary fiber (cellulose, pectin, inulin and *P. psyllium*) had a notable impact on the variable levels of Tau protein ($P<0.001$).

D × F interaction ($P<0.001$) was observed for this parameter and it resulted from the fact that applying a higher dosage of CuNPs in the CNH, PNH and JNH increased the percentage of DNA methylation, which was not observed in the SNH group. Moreover, a D × F interaction ($P = 0.016$) was observed for the βAP levels. This interaction was caused by the fact that applying a higher dosage of CuNPs in the CNH and SNH groups did not increase the levels of βAP in the hippocampus, while in the PNH and JNH groups the levels of this peptide were increased. Rising the levels of CuNPs significantly increased the levels of p-Tau regardless of the applied source of dietary copper. D × F interaction ($P<0.001$) for p-Tau resulting from applying a higher dose of copper increased the levels of p-Tau in the PNH and JNH groups, caused a decrease in the SNH group and in CNH group there were no significant changes of the levels of p-Tau (Figure 2). D × F interaction ($P = 0.024$) also concerned the level of CAMK2a in the hippocampus, and it resulted from the fact that the use of a higher dose of CuNPs in the CNH and SNH group caused a decrease in the level of this enzyme, which was not observed in the JNH and PNH groups.

Table 3. Hippocampal tissue parameters in rats fed experimental diets (n=8 per group)*

Treatment	LRP1, pg/mg	ABC, ng/mg	% mC	βAP, pg/mg	CAMK2α, ng/mg
Control C	3.95±0.248	0.893±0.055	78.4±1.027	23.4±0.702	0.553±0.053
Control CH	2.81±0.140	0.375±0.089	86.5±1.824	30.1±0.832	1.12±0.066
2-way ANOVA					
CN	6.07±0.134$^{\#}$	1.22±0.098 cd	75.8±0.464 bc$^{\#}$	16.2±0.703 a$^{\#}$	0.296±0.027 a$^{\#}$
CNH	6.09±0.163$^{\&}$	0.379±0.038 e	79.4±0.584 a$^{\&}$	16.2±0.382 a$^{\&}$	0.228±0.021 b$^{\&}$
PN	6.08±0.112$^{\#}$	1.32±0.130 c	75.5±0.402 c$^{\#}$	8.45±0.265 d$^{\#}$	0.180±0.022 bc$^{\#}$
PNH	6.7±0.183$^{\&}$	0.923±0.038 d$^{\&}$	79.3±0.494 a$^{\&}$	10.3±0.748 bc$^{\&}$	0.209±0.034 bc$^{\&}$
JN	6.09±0.141$^{\#}$	2.38±0.153 b	76.1±0.695 bc	8.36±0.567 d$^{\#}$	0.176±0.017 bc$^{\#}$
JNH	6.10±0.099$^{\&}$	1.48±0.160 c$^{\&}$	79.6±0.557 a$^{\&}$	11.5±0.358 b$^{\&}$	0.144±0.021 c$^{\&}$
SN	6.11±0.097$^{\#}$	2.86±0.122 a	77.3±0.602 b	9.91±0.302 c$^{\#}$	0.141±0.014 c$^{\#}$
SNH	6.17±0.182$^{\&}$	1.53±0.114 c$^{\&}$	76.7±0.434 bc$^{\&}$	11.2±0.239 bc$^{\&}$	0.038±0.006 d$^{\&}$
Cu-NP dose (D)					
N (6.5 g/kg)	6.09	1.95	76.2	10.7	0.198
H (13 mg/kg)	6.11	1.08	78.8	12.3	0.154
P value	0.831	<0.001	<0.001	<0.001	0.006
Fiber type (F)					
C (cellulose)	6.08	0.798	77.6	16.2	0.262
P (pectin)	6.08	1.12	77.4	9.36	0.194
J (inulin)	6.09	1.93	77.9	9.95	0.160
S (psyllium)	6.14	2.20	77.0	10.6	0.089
P value	0.967	<0.001	0.436	<0.001	<0.001
Interaction D×F					
P value	0.996	0.002	<0.001	0.016	0.024

*Control groups C and CH, fed a diet with standard and enhanced Cu content (6.5 and 13 mg/kg from $CuCO_3$, respectively). In the remaining groups, the diets were supplemented with copper nanoparticles (CuNPs) at two doses: 6.5 mg/kg (groups CN, PN, JN, SN) or 13 mg/kg (groups CNH, PNH, JNH, SNH). The dietary fiber in the diets: 8% of cellulose (groups C, CH, CN, CNH), 6% of pectin + 2% of cellulose (groups PN, PNH), 6% inulin + 2% cellulose (JN, JNH), 6% psyllium + 2% cellulose (SN, SNH).

a–e – mean values ±SEM within a column with unlike superscript letters are shown to be significantly different (P<0.05); differences among the groups fed diets with CuNPs are indicated only in the case of a significant interaction D×F. Additionally with the aid of Dunnett's test, each experimental group fed CuNPs 6.5 mg/kg or 13 mg/kg was compared with the control C or CH one, respectively (indicated by $^{\#}$ and $^{\&}$, respectively). SEM, standard error of mean (standard deviation for one group divided by the square root of rat number, n=8).

LRP1, LDL receptor related protein 1; ABC, ATP-binding cassette; % mC, DNA methylation percentage; βAP, β-amyloid, p-Tau, phosphorylated Tau protein; CAMK2α, Ca/calmodulin depend ent protein kinase II alpha.

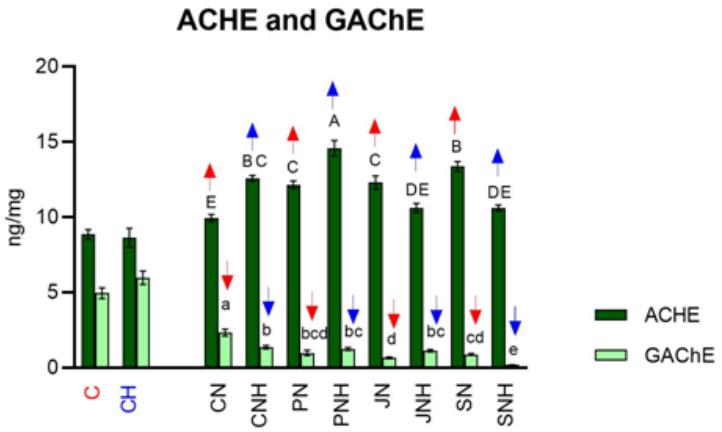


Figure 1. Acetylcholinesterase (AChE) and glycosylated AChE (GAChE) levels in the hippocampal tissue of rats fed experimental diets. $^{A-E}$ (for AChE) $^{or\ a-e}$ (for GAChE) Mean values with unlike superscript letters are shown to be significantly different (P<0.05). Additionally with the aid of Dunnett's test, each experimental group fed CuNPs 6.5 mg/kg or 13 mg/kg was compared with the control C or CH one, respectively (significant increase or decrease, indicated by red and blue arrows, respectively). Control groups C and CH, fed a diet with standard and enhanced Cu content (6.5 and 13 mg/kg from $CuCO_3$, respectively). In the remaining groups, the diets were supplemented with copper nanoparticles (CuNPs) at two doses: 6.5 mg/kg (groups CN, PN, JN, SN) or 13 mg/kg (groups CNH, PNH, JNH, SNH). The dietary fiber in the diets: 8% of cellulose (groups C, CH, CN, CNH), 6% of pectin + 2% of cellulose (groups PN, PNH), 6% inulin + 2% cellulose (JN, JNH), 6% psyllium + 2% cellulose (SN, SNH)

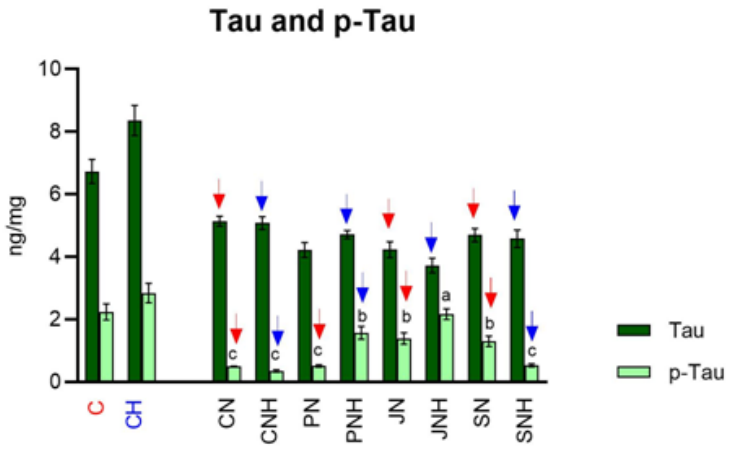


Figure 2. Tau protein (Tau) and phosphorylated Tau protein (p-Tau) levels in the hippocampal tissue of rats fed experimental diets. [a-c] (for p-Tau) Mean values with unlike superscript letters are shown to be significantly different (P<0.05). Additionally with the aid of Dunnett's test, each experimental group fed CuNPs 6.5 mg/kg or 13 mg/kg was compared with the control C or CH one, respectively (significant decrease indicated by red and blue arrows, respectively).

Control groups C and CH, fed a diet with standard and enhanced Cu content (6.5 and 13 mg/kg from $CuCO_3$, respectively). In the remaining groups, the diets were supplemented with copper nanoparticles (CuNPs) at two doses: 6.5 mg/kg (groups CN, PN, JN, SN) or 13 mg/kg (groups CNH, PNH, JNH, SNH). The dietary fiber in the diets: 8% of cellulose (groups C, CH, CN, CNH), 6% of pectin + 2% of cellulose (groups PN, PNH), 6% inulin + 2% cellulose (JN, JNH), 6% psyllium + 2% cellulose (SN, SNH)

Two-way ANOVA analysis showed Dose × Fiber type (D × F; P<0.001) interaction for AChE in the hippocampus, which resulted from the fact that regardless of the type of administered dietary fiber, applying a higher dosage of CuNPs in the CNH and PNH groups caused an increase of AChE, while in the JNH and SNH groups it caused a decrease of this enzyme (Figure 1). For GAChE the D × F interaction (P<0.001) was caused by increasing the dosage of CuNPs to 13 mg in the CNH and SNH groups, which increased the levels of GAChE in the hippocampus, while increasing the dosage of CuNPs in the JNH and SNH groups had no influence on the levels of GAChE in the hippocampus. However, increasing the dosage of CuNPs administered with the diet caused D × F interaction (P = 0.002) for the level of ABC (Table 3). It was caused by the fact that regardless of the applied source of dietary fiber the level of ABC in the hippocampus decreased in every group receiving CuNPs. Applying different sources of dietary fiber with simultaneous increase in the dosage of copper from 6.5 mg to 13 mg considerably influenced the epigenetic changes of DNA methylation in the hippocampal tissue. D × F interaction (P<0.001) was observed for this parameter and it resulted from the fact that applying a higher dosage of CuNPs in the CNH, PNH and JNH increased the percentage of DNA methylation in the hippocampus, which was not observed in the SNH group. Moreover, D × F interaction (P = 0.016) was observed for the βAP levels. This interaction was caused by the fact that applying a higher dosage of CuNPs in the CNH and SNH groups did not increase the levels of βAP in the hippocampus, while in the PNH and JNH groups the level of this peptide was increased. Rising the levels of CuNPs in diet significantly increased the levels of p-Tau in the rats' hippocampus regardless of the applied source of dietary copper. D × F interaction (P<0.001) for p-Tau resulted from applying a higher dose of copper and increased the levels of p-Tau in the PNH and JNH groups, caused a decrease in the SNH group and in CNH group there were no significant changes of the levels of p-Tau (Figure 2). D × F interaction (P = 0.024) also concerned the level of CAMK2a in the hippocampus, and it resulted from the fact that the use of a higher dose of CuNPs in the CNH and SNH group caused a decrease in the level of this enzyme, which was not observed in the JNH and PNH groups.

The level of analyzed indicators in the jejunum

Regardless of the administered copper dosage the addition of pectin (P), inulin (I), and *P. psyllium* (J) resulted in a favorable decrease in the levels of Tau protein in the rat jejunum. Furthermore, the Dunnett's analysis demonstrated that administering 6.5 mg of CuNPs increased the levels of AChE in all groups compared with group C (Figure 3).

However, the administration of 13 mg of CuNPs increased the levels of AChE as observed in groups PNH, JNH and SNH in comparison with group CH. The addition as 6.5 mg of CuNPs resulted in a decrease of GAChE in the jejunum of the animals from CN and SN groups, yet it has increased the level of GAChE in the JN group compared with the C group. Administration of 13 mg of CuNPs in CNH, JNH and SNH groups led to a significant decrease in the levels of GAChE compared with the CH group. Notably, a comparison of all groups with group C showed that an increased level of ABC could be observed only in the jejunum of rats from the SN group

(Table 4). Moreover, regardless of the kind of the administered dietary fiber, applying the dosage of 6.5 mg of CuNPs increased the levels of LRP1 compared with the C group. Similarly, in the case of administering 13 mg of CuNPs, the levels of LRP1 increased in all groups compared with the C group, and the kind of dietary fiber did not influence this parameter. However, a comparison with the CH group demonstrated an increase of ABC for CNH, PNH, JNH and SNH groups. Administering 13 mg of CuNPs led to lowered % mC for CNH, JNH and SNH groups compared with the CH group. The level of βAP decreased after administering 6.5 mg of CuNPs combined with pectin, inulin and *P. psyllium* compared with the C group. Nonetheless, administering 13 mg of CuNPs with any of the dietary fiber sources mentioned above decreased the levels of βAP in all groups compared with the CH group. The administration of 6.5 mg of CuNPs in addition with pectin or inulin or *P. psyllium* resulted in a decrease in the level of p-Tau and CAMP2a compared to group C (Figure 4; Table 4).

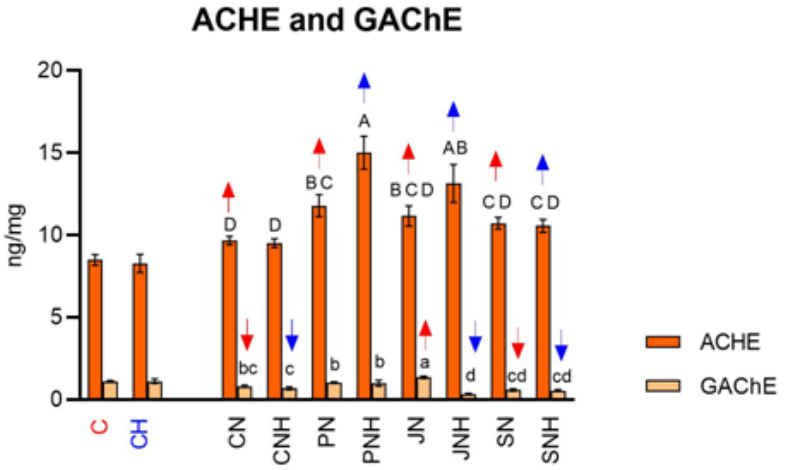


Figure 3. Acetylcholinesterase (AChE) and glycosylated AChE (GAChE) levels in the jejunal tissue of rats fed experimental diets. $^{A-D}$ (for AChE) or $^{a-d}$ (for GAChE) Mean values with unlike superscript letters are shown to be significantly different ($P<0.05$). Additionally with the aid of Dunnett's test, each experimental group fed CuNPs 6.5 mg/kg or 13 mg/kg was compared with the control C or CH one, respectively (significant increase or decrease, indicated by red and blue arrows, respectively).

Control groups C and CH, fed a diet with standard and enhanced Cu content (6.5 and 13 mg/kg from $CuCO_3$, respectively). In the remaining groups, the diets were supplemented with copper nanoparticles (CuNPs) at two doses: 6.5 mg/kg (groups CN, PN, JN, SN) or 13 mg/kg (groups CNH, PNH, JNH, SNH). The dietary fiber in the diets: 8% of cellulose (groups C, CH, CN, CNH), 6% of pectin + 2% of cellulose (groups PN, PNH), 6% inulin + 2% cellulose (JN, JNH), 6% psyllium + 2% cellulose (SN, SNH)

Table 4. Small intestinal tissue parameters in rats fed experimental diets (n=8 per group)*

Treatment	LRP1, pg/mg	ABC, ng/mg	% mC	βAP, pg/mg	CAMK2α, ng/mg
1	2	3	4	5	6
Control C	4.08±0.263	1.01±0.051	75.5±0.832	10.9±0.596	1.61±0.191
Control CH	2.51±0.202	0.568±0.067	87.7±2.044	15.9±1.086	2.32±0.233
		2-way ANOVA			
CN	6.07±0.194$^{\#}$	0.976±0.113 d	75.4±0.519 c	9.58±0.566 a	0.181±0.042 bc$^{\#}$
CNH	6.09±0.272$^{\&}$	1.98±0.046 a$^{\&}$	79.5±0.399 b$^{\&}$	10.5±0.558 a$^{\&}$	0.979±0.049 a$^{\&}$
PN	6.09±0.087$^{\#}$	1.08±0.094 cd	75.7±0.834 c	5.59±0.335 c$^{\#}$	0.983±0.040 a$^{\#}$
PNH	6.18±0.122$^{\&}$	1.29±0.067 bc$^{\&}$	85.4±1.074 a	10.6±0.228 a$^{\&}$	0.136±0.025 bcd$^{\&}$
JN	6.07±0.151$^{\#}$	0.853±0.062 d	75.6±0.343 c	4.14±0.196 d$^{\#}$	0.093±0.009 cd$^{\#}$
JNH	6.12±0.303$^{\&}$	0.856±0.158 d$^{\&}$	79.4±0.839 $^{b\&}$	7.11±0.298$^{b\&}$	0.206±0.055 $^{b\&}$
SN	6.18±0.269$^{\#}$	1.39±0.123 b$^{\#}$	75.5±0.714 c	5.73±0.373 c$^{\#}$	0.080±0.010 cd$^{\#}$
SNH	6.22±0.118$^{\&}$	1.91±0.069 a$^{\&}$	74.8±0.591 c$^{\&}$	6.51±0.289 bc$^{\&}$	0.063±0.006 d$^{\&}$
		Cu-NP dose (D)			
N (6.5 g/kg)	6.10	1.08	75.6	6.26	0.334
H (13 mg/kg)	6.15	1.51	79.8	8.69	0.346
P value	0.729	<0.001	<0.001	<0.001	0.639

Table 4. – contd.

1	2	3	4	5	6
			Fiber type (F)		
C (cellulose)	6.08	1.48	77.5	10.0	0.580
P (pectin)	6.13	1.19	80.6	8.12	0.560
J (inulin)	6.09	0.855	77.5	5.62	0.149
S (psyllium)	6.20	1.65	75.1	6.12	0.071
P value	0.932	<0.001	<0.001	<0.001	<0.001
			Interaction D×F		
P value	0.999	<0.001	<0.001	<0.001	<0.001

*Control groups C and CH, fed a diet with standard and enhanced Cu content (6.5 and 13 mg/kg from $CuCO_3$, respectively). In the remaining groups, the diets were supplemented with copper nanoparticles (CuNPs) at two doses: 6.5 mg/kg (groups CN, PN, JN, SN) or 13 mg/kg (groups CNH, PNH, JNH, SNH). The dietary fiber in the diets: 8% of cellulose (groups C, CH, CN, CNH), 6% of pectin + 2% of cellulose (groups PN, PNH), 6% inulin + 2% cellulose (JN, JNH), 6% psyllium + 2% cellulose (SN, SNH).

a–e – mean values ± SEM within a column with unlike superscript letters are shown to be significantly different (P<0.05); differences among the groups fed diets with CuNPs are indicated only in the case of a significant interaction D×F. Additionally with the aid of Dunnett's test, each experimental group fed CuNPs 6.5 mg/kg or 13 mg/kg was compared with the control C or CH one, respectively (indicated by # and &, respectively). SEM, standard error of mean (standard deviation for one group divided by the square root of rat number, n = 8).

LRP1, LDL receptor related protein 1; ABC, ATP-binding cassette; % mC, DNA methylation percentage; βAP, β-amyloid, CAMK2α, Ca/calmodulin dependent protein kinase II alpha.

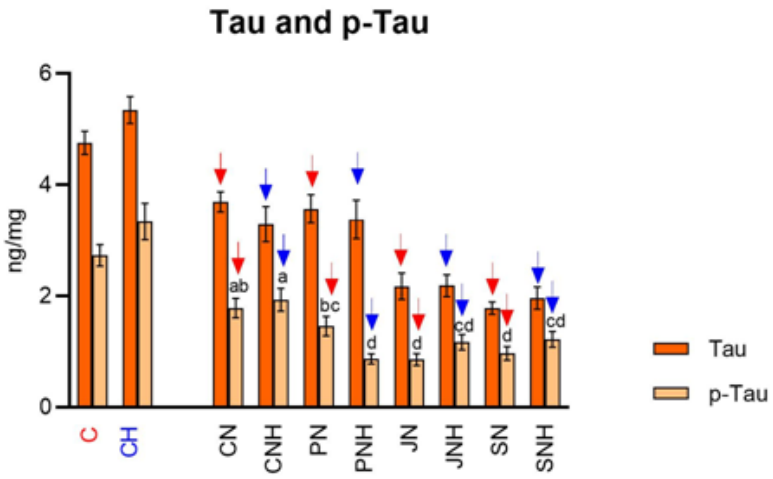


Figure 4. Tau protein (Tau) and phosphorylated Tau protein (p-Tau) levels in the jejunal tissue of rats fed experimental diets. [a-d] (for p-Tau) Mean values with unlike superscript letters are shown to be significantly different (P<0.05). Additionally with the aid of Dunnett's test, each experimental group fed CuNPs 6.5 mg/kg or 13 mg/kg was compared with the control C or CH one, respectively (significant decrease indicated by red and blue arrows, respectively).

Control groups C and CH, fed a diet with standard and enhanced Cu content (6.5 and 13 mg/kg from $CuCO_3$, respectively). In the remaining groups, the diets were supplemented with copper nanoparticles (CuNPs) at two doses: 6.5 mg/kg (groups CN, PN, JN, SN) or 13 mg/kg (groups CNH, PNH, JNH, SNH). The dietary fiber in the diets: 8% of cellulose (groups C, CH, CN, CNH), 6% of pectin + 2% of cellulose (groups PN, PNH), 6% inulin + 2% cellulose (JN, JNH), 6% psyllium + 2% cellulose (SN, SNH)

Moreover, a comparison with the CH group showed that the administration of 13 mg of CuNPs decreased the levels of p-Tau and CAMP2a. Two-way ANOVA analysis also showed an interaction of Cu-NP Dose × Fiber type (D × F; P = 0.033) regarding the level of AChE in the intestine tissue, which resulted from the fact that the use of a higher dose of CuNPs (13 mg) in the PNH group resulted in an increase in the level of this enzyme, which was not found in CNH, JNH and SNH treatments (Figure 3). An interaction D × F (P<0.001) was also observed for the GAChE level. This interaction stemmed from the fact that the use of a higher dose of copper in the form of nanoparticles in the CNH, PNH and SNH groups did not increase the level of GAChE, while increasing the share of nanoparticles in the JNH group resulted in a decrease in the level of GAChE in the intestine of rats. D × F interaction (P<0.001) noted for the ABC level was due to the fact that the use of a higher dose of copper nanoparticles in the PNH and JNH groups did not increase the ABC level, but increasing the dose of nanocopper to 13 mg in the CNH and SNH groups led to an increase in ABC in the intestine of rat (Table 4). In the analyzed tissue,

there was also D × F interaction (P<0.001) for the degree of DNA methylation, which resulted from the fact that the use of a higher dose of copper in the form of nanoparticles in the CNH, PNH and JNH groups resulted in an increase in the percentage of DNA methylation in the intestinal tissue. In the case of the SNH procedure, no such relationship was observed. D × F interaction (P<0.001) observed in the intestinal tissue for the β-amyloid level stemmed from the fact that the use of a higher dose of CuNPs in the CNH and SNH groups did not increase the level of this toxic peptide, but the increase in the dose of nanocopper from 6.5 to 13 mg in the PNH and JNH groups led to an increase in the concentration of β-amyloid in the analyzed tissue. There was an interaction D × F (P<0.001) for the level of p-Tau, which resulted from the fact that the use of a higher dose of copper in the form of nanoparticles in the PNH group caused a significant decrease of p-Tau level, while in the rats of the CNH, JNH and SNH treatments there was no change in the level of this indicator (Figure 4). D × F interaction (P<0.001) was found for the level of CAMK2a in the small intestinal tissue, and it resulted from the fact that the use of a higher dose of copper in the form of nanoparticles in the CNH and JNH groups resulted an increase of CAMK2a, and in rats from the PNH and SNH treatments, a decrease of CAMK2a. Two-way ANOVA analysis showed that the use of different sources of dietary fiber (cellulose or pectin or inulin or *P. psyllium*) clearly influenced the differentiation of the level of toxic Tau protein in the rat's jejunum (P<0.001) (Figure 4). Regardless of the dose of nano-copper used, the addition of inulin (J) and *P. psyllium* (S) resulted in a decrease in the concentration of Tau protein in the intestine of rats.

Two-way ANOVA analysis also showed an interaction of Cu-NP Dose × Fiber type (D × F; P = 0.033) regarding the level of AChE in the rats' intestine tissue, which resulted from the fact that the use of a higher dose of CuNPs (13 mg) in the PNH group resulted in an increase in the level of this enzyme, which was not found in CNH, JNH and SNH treatments (Figure 3). An interaction D × F (P<0.001) was also observed for the GAChE level. This interaction stemmed from the fact that the use of a higher dose of copper in the form of nanoparticles in the CNH, PNH and SNH groups did not increase the level of GAChE, while increasing the share of nanoparticles in the JNH group caused a decrease in the level of GAChE in the intestine of rats. D × F interaction (P<0.001) noted for the ABC level was due to the fact that the use of a higher dose of copper nanoparticles in the PNH and JNH groups did not increase the ABC level, but increasing the dose of nanocopper to 13 mg in the CNH and SNH groups led to an increase in ABC in the intestine of rat (Table 4). In the rat's jejunum, there was also D × F interaction (P<0.001) for the degree of DNA methylation, which resulted from the fact that the use of a higher dose of copper in the form of nanoparticles in the CNH, PNH and JNH groups caused an increase in the percentage of protein methylation in DNA. D × F interaction (P<0.001) observed in the intestinal tissue for the β-amyloid level stemmed from the fact that the use of a higher dose of CuNPs in the CNH and SNH group did not increase the level of this toxic peptide, but the increase in the dose of nanocopper from 6.5 to 13 mg in the PNH and JNH groups led to an increase the concentration of β-amyloid in the rats' jejunum. There was an interaction D × F (P<0.001) for the level of p-Tau was also found, which resulted from the fact that the use of a higher dose of copper in the form of nanoparticles in the PNH group caused a significant decrease of p-Tau level, while in the rats of the CNH, JNH and SNH treatments there was no change in the level of this indicator (Figure 4). D × F interaction (P<0.001) was found for the level of CAMK2a in the small intestinal tissue, and it resulted from the fact that the use of a higher dose of copper in the form of nanoparticles in the CNH and JNH group caused an increase, and in rats from the PNH and SNH treatments, a decrease in the level of this enzyme. Two-way ANOVA analysis showed that the use of different sources of dietary fiber (cellulose or pectin or inulin or *P. psyllium*) clearly influenced on the differentiation of the level of toxic Tau protein in jejunum (P<0.001) (Figure 4). Irrespective of the dose of nanocopper used, the addition of inulin (J) and *P. psyllium* (S) resulted in a decrease in the concentration of Tau protein in the rat intestine.

Discussion

The results of our own research clearly indicate the varied effect of replacing copper carbonate with copper nanoparticles (6.5 mg and 13 mg) and nanocopper (6.5 mg and 13 mg) in combination with various types of dietary fiber (cellulose, pectin, inulin and *P. psyllium*) on neurometabolism in the hippocampus and intestines of rats.

Our findings indicate that replacing the inorganic form of copper with CuNPs in the amount of 6.5 mg or 13 mg can significantly boost the level of LRP1, in both the rats' hippocampus and intestine. According to Cendrowska-Pinkosz et al. (2021) the addition of nanocopper may contribute to an increase in the concentration of LRP1 in rat tissues. Citing the findings of Cendrowska-Pinkosz et al. (2021), it has been suggested that the excessive production of the APP protein, a βAP precursor that helps to lower LRP1 levels, may also be a contributing factor to neurodevelopmental abnormalities. Analyzing the results obtained in the present research, regarding the replacement of $CuCO_3$ with nanoparticles, it can be concluded that the level of β-amyloid in the hippocampal and jejunal tissue might be reduced with the aid of dietary nanocopper added to a diet at both doses (6.5 or 13 mg/kg of a diet). Such an effect of copper nanoparticles in the context of replacing the cationic form of copper carbonate with them should be considered beneficial from the point of view of limiting neurodevelopmental changes and supporting neurometabolism.

According to Cendrowska-Pinkosz et al. (2021), copper cations can accumulate in the βAP plaques and increase their production. Additionally, copper cations easily bind with p-Tau, which is responsible for fibril degeneration. What is more, βAP itself can stimulate the formation of neurotoxic clusters of p-Tau. This process is crucial for the mechanism of microglia activation by Tau protein and βAP plaques, which initially results in the removal of those neurotoxic clusters. However, the continuous process of Tau protein and βAP aggregation initiates neural inflammation, which leads to pathological processes such as the formation of neurotoxic βAP and Tau protein clusters, p-Tau synthesis as well a decline in the neuron renewal process (Maynard et al., 2005). The problem of amyloid deposition begins from the very beginning of the rat's growth and development, i.e., immediately after birth. According to the same authors, at a later age, i.e., around 6 months of age, pathological deposition and accumulation of βAP oligomers begins. It is similar with the Tau protein, the level of which increases from the first week of life, reaching a significantly high level at the age of approximately 3 months (8–12 weeks of life) (Orr et al., 2015). A surprising result regarding p-Tau was found in the intestine of rats from some groups (CN, CNH, PN, SNH), where higher levels of p-Tau were observed than in the hippocampal tissue. Gu et al. (1996), Dugger et al. (2016), Chambonnière et al. (2001) and Tam (1999) have shown that tau is expressed in rodent and human gastrointestinal (GI) tract, but no data are available about the distribution and phosphorylation pattern of tau isoforms in the enteric nervous system (ENS). The enteric nervous system shows some unique features that closely resemble the CNS and is sometimes referred to as the 'brain-in-the-gut' or the 'second brain'. This close homology between the CNS and ENS suggests that a disease process affecting the CNS could also involve its enteric counterpart (Lionnet et al., 2018).

In the case of using CuNPs as the only source of copper, combined with various types of dietary fiber, a beneficial reduction in the level of βAP in the hippocampus and small intestine of rats was found. Such an effect was shown by a dose of 6.5 mg of CuNPs combined with any type of fiber and a dose of 13 mg, but only when combined with cellulose or *P. psyllium*. In the study by Poulson et al. (2020) the authors claimed that dietary cellulose has neuroprotective properties. They observed that dietary cellulose decreased the hydrophobicity of peptides and proteins (including βAP) in the neurons, and as a result, it prevents neuronal fibrosis. Stabilizing protein structures decreases their aggregation, which prevents the formation of βAP aggregates and the formation of Tau protein neurofibrillary tangles. The antioxidative properties of fiber decrease the overproduction of pro-inflammatory free radicals in the neurons, thus preventing changes in neurometabolism by creating toxic peptide aggregates (Poulson et al., 2020). Uddin et al. (2020) stated that the abnormal protein and peptide folding causes oligomers and clusters of Tau protein, p-Tau, and βAP to appear. As a result, said clusters disturb neurometabolism and stimulate the rise of neurodegenerative changes. According to Wang et al. (2021) the neuroprotective properties of cellulose result from its nutritional effect on the nervous tissue, which improves neurogenesis. Muneer et al. (2017) stated that cellulose increases the expression of β-catenin which regulates angiogenesis in the nervous system and stimulates the synthesis of brain-derived neurotrophic factor (BDNF) in the hippocampus. Yamagishi et al. (2023) reported that cellulose can mitigate neurodegeneration-related nerve inflammation and decrease the accumulation of βAP in the brain through intestinal bacteria which synthesize butter acid (as a result of dietary fiber fermentation, short-chain fatty acids, and phytosterols). McRorie (2015), Shah et al. (2020) and Chen et al. (2022) explain the improvement of neurometabolism by the unique viscous potential of *Plantago psyllium*, which refines the gut microbiome, has antibacterial properties and reduces the levels of cholesterol and glucose in the blood. However, Poulson et al. (2020) believe that cellulose might stimulate neurometabolism by stimulating the function of the immune system, decreasing insulin resistance, enhancing glucose metabolism, and regulating the activity of erythrocytes. Cellulose takes part in the epigenetic control of expression of the genes that control metabolic and tissue homeostasis of glucose, cholesterol, and βAP. For βAP, this process occurs due to the regulation of histone deacetylase (HDAC). This phenomenon is most intense in the cortex and hippocampus and is accompanied by an increase in cholesterol levels in those tissues (Cendrowska-Pinkosz et al., 2022). According to Fernando et al. (2018), Yamagishi et al. (2023) and Ferenc et al. (2023) dietary fiber increases both the synthesis and metabolism of acetylcholine by stimulating the metabolic activity of acetylcholinesterase. Arabinoxylans are responsible for stabilizing the structure of *Plantago psyllium* as they display strong protein-binding properties through tyrosine or cysteine. Additionally, by binding with ferulic acid, they gain antioxidative properties (Chen et al., 2022). Patel and Aschner (2021) concluded that water retaining and gel-producing abilities of *Plantago psyllium* in the gut improve the enzymatic activity of the gut microbiome. It is particularly beneficial for bacteria of the genus *Bacteroides*, which leads to improved metabolism of cholesterol. A disruption in cholesterol metabolism is a partial cause of neurometabolic changes. Cholesterol is the crucial ingredient of the myelin sheaths of neurons. They function correctly when cholesterol can attack LRP1, which is responsible for the proper clearance of βAP (Dhahri et al., 2022). Rauch et al. (2020) claims that LRP1 also captures Tau and p-Tau protein oligomers. In our own research, replacing $CuCO_3$ with nanoparticles of this element (6.5 mg and 13 mg) allowed for a very beneficial increase in the level of AChE in the hippocampus and small intestine of rats. At the same time, in the case of an attempt to combine an increased dose of CuNPs (13 mg) with various types of dietary fiber, it turned out that the most beneficial level of

this enzyme was increased by a dietary combination with pectin. Increasing the level of AChE in the brain and intestine of the rats studied should be considered a beneficial phenomenon, since AChE deficiency is associated with cholinergic dysfunction in neurons, excessive accumulation of acetylcholine and the appearance of neuroinflammatory changes (Underhill and Amara, 2021). According to Underhill and Amara (2021) the excessive increase of the acetylcholine concentration enhances protein phosphorylation, such as Tau protein phosphorylation (Nuzzo et al., 2021). On the other hand, AChE is the main protein of cholinergic synapses of the brain and neuromuscular connections, therefore a decrease in the levels of AChE could be a sign of early neuropathologies. This phenomenon is most intense in the cortex and hippocampus and is accompanied by an increase in cholesterol levels in those tissues (Cendrowska-Pinkosz et al., 2021). An important cause of neurological ageing and neurodegeneration changes is also the possibility of accumulation of large amounts of copper in the amyloid plaques themselves (Bagheri et al., 2018; Cendrowska-Pinkosz et al., 2021) which according to Maynard et al. (2005) further increases the toxicity of amyloid-β for neuronal cells. According to Nuzzo et al. (2021) pectin has a very beneficial effect on the structure and metabolism of nervous tissue. It inhibits lipid peroxidation of neuronal membranes of mitochondria (Nuzzo et al., 2021), has a neurotrophic effect and inhibits the toxic effects of galectin-3, responsible for the pathogenesis of metabolic disorders and the development of oxidative stress (Nuzzo et al., 2021; Maynard et al., 2005). Cui et al. (2022) think that increase of AChE, a major protein of brain cholinergic synapses and neuromuscular junctions, limits the initiation of early neuropathological changes. The research results of Cui et al. (2022) indicate that AChE is the main protein of brain cholinergic synapses and neuromuscular junctions limiting the initiation of early neuropathological changes. Although in our studies the use of 13 mg of CuNPs and 2% share of pectin in the diet significantly increased the level of AChE, in our opinion, the dose of 6.5 mg of CuNPs is more favorable, due to the fact that the increasing excess of AChE may, unfortunately, promote the development of neurometabolic changes (Fatma et al., 2011). Moreover, Dhahri et al. (2022) showed that excess AChE is associated with cholinergic dysfunction in neurons, increased hydrolysis of acetylcholine and the appearance of neuroinflammatory changes. Replacing $CuCO_3$ with CuNPs also very favorably reduced the level of GAChE in the hippocampus, regardless of the dose of nanoparticles used. Also, the combination of 13 mg of CuNPs with cellulose or *P. psyllium* allowed reducing the concentration of this complex in this tissue. The reduced level of GAChE found in the hippocampus of rats receiving CuNPs (in place of the inorganic form of copper), observed in our own research, suggests that the effect of copper in the nano form is more neuroprotective than copper in the cation form, because CuNPs, better than copper in the inorganic form, limits the formation of enzymatically inactive complexes of β-amyloid with acetylcholinesterase and Tau protein (GAChE). Therefore, CuNP is most likely to be responsible for those events, which confirms the results obtained by Cendrowska-Pinkosz et al. (2021). Underhill and Amara (2021) demonstrated that the formation of GAChE promotes mitochondrial dysfunction and increases the formation of ROS, which induces neural apoptosis.

As reported by Krauze et al. (2022) the neurometabolic changes caused by both amyloid-β and GAChE itself result in the formation of large amounts of reactive oxygen species, especially in the presence of Ca^{2+} ions. The mechanism of neurometabolic disruption is due to NMDA glutamatergic receptor activation, calcium channel opening, Ca^{2+} influx and CAMK2a activation. This process causes the overproduction of free radicals and oxidative changes in the cellular membrane of neurons (Emmett et al., 2004). The excessive concentration of Ca^{2+} within the cell decreases the pace of oxidative phosphorylation and ATP production, as well as promotes the opening of the mitochondrial megachannels, which results in the malfunction of neurometabolism (Nuzzo et al., 2021). In our own research, a very promising reduction in the level of CAMP2a in the hippocampus and intestine of rats was found after administration of both 6.5 mg and 13 mg of CuNPs, instead of the inorganic form of this element. At the same time, reducing the level of this enzyme in the hippocampus and in the small intestinal tissue was obtained after using 13 mg of CuNPs in the diet in combination with pectin or *P. psyllium*. CAMP2a is responsible for the formation of calmodulin-calcium complexes, which may lead to excessive accumulation of this ion in the mitochondria and to disruption of oxidative phosphorylation. Excessive influx of Ca^{2+} into the mitochondrion induces a number of pathological changes resulting from oxidative stress. The result is a reduction in ATP production, disruption of neurometabolism in neurons, and slow neuronal cell death. In our researches we also found that the using of 13 mg of CuNPs in combination with inulin caused a significantly decrease of level the GAChE in the intestinal tissue. Ansari and Emerald (2019) showed that nutritional modulation with inulin significantly improves the functioning of the gut-brain axis. Inulin, protecting the intestinal mucus layer and having a neurotrophic effect, improves the metabolic functions of enterocytes (Schroeder et al., 2018). In addition, it promotes the formation of butyric acid in the intestines which plays a key role in modulating neurometabolism (Yanckello et al., 2022) and increases the adhesion of the microbiome to the intestinal wall.

Barclay and Petrovsky (2016) claim that inulin combined with nanoparticles gains adjuvant properties which reinforce the humoral and cellular response towards bacteria and viruses as well as toxins and allergens. Similar results have been obtained by Ferrando et al. (2021) who investigated the properties of inulin distributed with gold nanoparticles. Santillán-Urquiza et al. (2015) believe that the combination of nanoparticles with inulin in-

creases the bioavailability of this element in the digestive tract. We observed also that by using 13 mg of CuNPs (instead of the inorganic form of Cu) the level of ABC in the intestine of rats can be beneficially increased. In the case of using different sources of fiber, such an effect was obtained only in the case of a dietary combination with cellulose or with *Plantago psyllium*. ABC transporters responsible for the transport of ATP in the neuron are strongly involved in maintaining proper homeostasis in the brain by eliminating toxic peptides such as amyloid-β, Tau protein or p-Tau, which accumulate in the brain during the neurodevelopmental disorders (Michalicova et al., 2020; Mello et al., 2020). The highest level of expression of phosphorylated tau protein occurs in the early period of development, i.e., at the age of approximately 3 months (Orr et al., 2015). According to Tamaki et al. (2006), both LRP-1 expression and amyloid-B uptake are significantly lower in 13-month-old rats compared to 7-week-old rats. It was observed that the use of 13 mg of CuNPs in place of $CuCO_3$, might promisingly reduce the degree of DNA methylation in the intestinal tissue, i.e., epigenetic changes resulting from the covalent addition of a methyl group to the carbon at position 5 in the cytosine in the DNA molecule. Also, this dose of CuNPs (13 g) combined with *P. psyllium*, most strongly reduced the percentage of methylation in the intestine of the tested rats. Changes may arise as a result of covalent modifications of lysine and arginine residues in DNA, and an increased degree of methylation would suggest silencing of DNA repair genes. The effect of such a reaction may be to direct the cell into the apoptotic pathway (Cui et al., 2022). The reduction in the degree of methylation should therefore be considered a positive phenomenon, demonstrating the absence of disturbances in the DNA methylation pathways, causing disturbances at the level of the activity of individual enzymes responsible for the functioning of the nervous system (Mello et al., 2020). The use of 6.5 mg and 13 mg of Cu nanoparticles (especially in combination with inulin and *P. psyllium*) instead of the carbonate form of this element, resulted in a very beneficial reduction in the level of Tau protein. This is very promising from the point of view of reducing neurodevelopmental disorders. The results of our analyses also showed that replacing the carbonate form of copper with nanoparticles, regardless of the dose of CuNPs (6.5 mg and 13 mg), can very promisingly inhibit the formation of p-Tau in the hippocampal and small intestinal tissue of rats. Among the types of dietary fiber used, *P. psyllium* seems to be the best solution, because its administration together with 13 mg of CuNPs resulted in a decrease in p-Tau in the hippocampus of rats and no increase in the level of this indicator in the intestinal tissue. Tau protein promotes Aβ-induced neurotoxicity. Tau aggregation can be affected by post-translational modifications, in particular phosphorylation, also leading to the formation of phosphorylated p-Tau, and post-translational methylation also promoting Tau aggregation. Phosphorylated Tau protein can damage the cytoskeleton of neurons, thus contributing to disturbances in the transport of nutrients in these cells and the transmission of electrical signals between neurons in synapses (Krauze et al., 2023; Gao et al., 2022). Hyperphosphorylation of the Tau protein disrupts the interactions between the Tau protein (the physiological amount of which is needed to stabilize the cytoskeleton in neurons) and microtubules. In the opinion of Voss et al. (2014), binding of copper by β-amyloid induces spontaneous accumulation of this peptide molecules and the formation of Tau protein tangles and phosphorylation of Tau protein at a rate faster than in the absence of this metal. Exposure to excess Cu^{2+} can initiate and exacerbate Tau protein hyperphosphorylation and Tau protein cluster formation, contributing to synaptic failure and neuronal death (Zubčić et al., 2020; Šimić et al., 2022). Ca^{2+} plays a significant role in the development of neurometabolic disruption due to the fact that it contributes to the misfolding of the Tau protein and is dominant in the neurofibrillary tangles of the Tau protein (Mayes et al., 2014; Rossi et al., 2004).

Conclusions

By replacing copper carbonate in the diet of rats with copper in the form of nanoparticles, both at a dose of 6.5 mg and 13 mg of CuNPs, you can beneficially limit metabolic disorders that may generate neurodevelopmental and epigenetic changes in hippocampal and jejunal tissue. Partially replacing cellulose (which is a source of dietary fiber) by fiber from *Plantago psyllium* allows the beneficial neuroprotective effect of copper nanoparticles to be enhanced. Although the beneficial neuroprotective effect was shown by both doses of copper nanoparticles, and their effect was enhanced by the use of fiber from *Plantago psyllium*, due to the cost of using copper nanoparticles, it is worth recommending their lower dose.

Conflicts of interest

The authors declare no conflict of interest.

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