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lek. wet. Aleksandra Agnieszka Marzec

Rozprawa doktorska

Czy odpowiedź immunologiczna i oksydoredukcyjna organizmu na zastosowanie nanocząstek miedzi zależna jest od zróżnicowanych funkcji fizjologicznych błonnika pokarmowego?

Does the immunological and redox response of the organism to copper nanoparticle supplementation depend on the distinct physiological functions of dietary fiber?

Rozprawa doktorska wykonana w Katedrze Biochemii i Toksykologii

Promotor: prof. dr hab. Katarzyna Ognik
Promotor pomocniczy: dr hab. Bartosz Fotschki

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Serdeczne podziękowania składam

Pani Promotor prof. dr hab. Katarzynie Ognik

oraz Panu Promotorowi pomocniczemu dr hab. Bartoszowi Fotschki

Dziękuję serdecznie całemu zespołowi zaangażowanemu w projekt.

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Data 21.05.2026 Podpis autora Aleksandra Mazur

Wykaz prac naukowych wchodzących w skład cyklu

1. Cholewińska E., **Marzec A**., Sołek P., Fotschki B., Listos P., Ognik K., Juśkiewicz J. **2023**. The effect of copper nanoparticles and a different source of dietary fibre in the diet on the integrity of the small intestine in the rat. *Nutrients* 15, 7: 1588, DOI: 10.3390/nu15071588

   MNiSW = 140; IF = 4,800

2. **Marzec A**., Fotschki B., Napiórkowska D., Fotschki J., Cholewińska E., Listos P., Juśkiewicz J., Ognik K. **2024**. The effect of copper nanoparticles on liver metabolism depends on the type of dietary fiber. *Nutrients* 16, 21: 3645, DOI: 10.3390/nu16213645

   MNiSW = 140; IF = 5,000

3. **Marzec A**., Cholewińska E., Fotschki B., Juśkiewicz J., Ognik K. **2025**. Inulin improves the redox response in rats fed a diet containing recommended copper nanoparticle (CuNPS) levels, while pectin or psyllium in rats receive excessive CuNPS levels in the diet. *Antioxidants* 14, 6: 695, DOI: 10.3390/antiox14060695

   MNiSW = 100; IF = 6,600

4. **Marzec A**., Cholewińska E., Fotschki B., Juśkiewicz J., Stępniowska A., Ognik K. **2025**. Are the biodistribution and metabolic effects of copper nanoparticles dependent on differences in the physiological functions of dietary fibre? *Annals of Animal Science* 25, 1: 175 - 187, DOI: 10.2478/aoas-2024-0057

   MNiSW = 140; IF = 2,200

5. **Marzec A**., Fotschki B., Cholewińska E., Dworzański W., Juśkiewicz J., Ognik K. **2026**. Is the impact of copper nanoparticles on the immune system of rats dependent on the diverse physiological functions of dietary fibre? *Journal of Animal and Feed Sciences* e32. DOI: 10.22358/jafs/215745/2026.

   MNiSW = 100; IF = 1,500

Suma MNiSW = 620; IF = 20,100

Spis treści

Wykaz skrótów i akronimów

APE-1 — endonukleaza apurynowo/apirymidynowa 1

CAT — katalaza

cDNA — komplementarny DNA

COX-2 — cyklooksygenaza 2

Cp — ceruloplazmina

CRP — białko C-reaktywne

CTR1 — transporter miedzi 1

CuNPs / Cu-NP — nanocząstki miedzi

CuZn-SOD — dysmutaza ponadtlenkowa miedziowo-cynkowa

DMT1 — transporter metali dwuwartościowych 1

DNA — kwas deoksyrybonukleinowy

EDTA — kwas etylenodiaminotetraoctowy

ELISA — test immunoenzymatyczny

GSH — glutation zredukowany

H&E — hematoksylina i eozyna

HCT — hematokryt

HGB — hemoglobina

IgA — immunoglobulina A

IgG — immunoglobulina G

IgM — immunoglobulina M

IL-2 — interleukina 2

IL-6 — interleukina 6

LYM — limfocyty

MASLD — metabolicznie uwarunkowana stłuszczeniowa choroba wątroby

MCH — średnia masa hemoglobiny w krwince czerwonej

MCHC — średnie stężenie hemoglobiny w krwince czerwonej

MCV — średnia objętość krwinki czerwonej

MDA — dialdehyd malonowy

MPV — średnia objętość płytek krwi

NEU — neutrofile

NF-κB — jądrowy czynnik κB

PC — karbonylowe pochodne białek

PCR — reakcja łańcuchowa polimerazy

PDWc — wskaźnik zróżnicowania objętości płytek krwi

PLT — płytki krwi

PPAR-γ — receptor aktywowany przez proliferatory peroksysomów gamma

PCT — płytkokryt

qPCR — ilościowa reakcja łańcuchowa polimerazy w czasie rzeczywistym

RBC — erytrocyty

RDWc — wskaźnik zróżnicowania objętości erytrocytów

RNA — kwas rybonukleinowy

ROS — reaktywne formy tlenu

SCFA — krótkołańcuchowe kwasy tłuszczowe

SOD — dysmutaza ponadtlenkowa

SREBP-1c — białko wiążące element sterolowy 1c

TAS — całkowity status antyoksydacyjny

TLR-4 — receptor Toll-podobny 4

TNF-α — czynnik martwicy nowotworów alfa

WBC — leukocyty

Streszczenie

Celem podjętych badań było określenie, czy odpowiedź immunologiczna oraz oksydoredukcyjna organizmu na suplementację nanocząsteczek miedzi (CuNPs) zależy od właściwości fizjologicznych błonnika pokarmowego. Postawiono hipotezę, że działanie CuNPs ma charakter zależny od dawki i jest modulowane przez rodzaj błonnika, który poprzez wpływ na środowisko jelitowe i mikrobiotę może regulować ich biodostępność oraz efekt metaboliczny.

Badania przeprowadzono na szczurach Wistar w układzie dwuczynnikowym, obejmującym dwie dawki miedzi (6,5 i 13 mg/kg), dwie jej formy ($CuCO_3$ i CuNPs) oraz cztery rodzaje błonnika (celuloza, inulina, pektyna, psyllium). Analizowano wpływ tych czynników na funkcjonowanie przewodu pokarmowego, status oksydacyjno-redukcyjny, biodostępność i dystrybucję miedzi, metabolizm wątrobowy oraz odpowiedź immunologiczną.

Wyniki wykazały, że CuNPs charakteryzują się wyższą biodostępnością niż tradycyjne formy nieorganiczne, co prowadzi do zmiany ich dystrybucji w organizmie oraz wpływa na homeostazę innych pierwiastków. Jednocześnie CuNPs mogą indukować stres oksydacyjny i modulować odpowiedź immunologiczną, przy czym efekt ten jest zależny od dawki. Wykazano, że jelito odgrywa kluczową rolę w regulacji odpowiedzi organizmu na CuNPs. Oddziaływanie nanocząsteczek z mikrobiotą jelitową wpływa na produkcję krótkołańcuchowych kwasów tłuszczowych, integralność bariery jelitowej oraz procesy zapalne, determinując dalsze efekty ogólnoustrojowe.

Istotnym czynnikiem modulującym działanie CuNPs okazał się błonnik pokarmowy. Inulina wykazywała działanie ochronne przy dawce rekomendowanej, natomiast pektyna i psyllium ograniczały stres oksydacyjny i odpowiedź zapalną przy wyższej podaży CuNPs. Różnice te wynikają z odmiennych właściwości fizykochemicznych błonnika.

Podsumowując, efekt działania CuNPs zależy od interakcji pomiędzy dawką nanocząsteczek a składem diety. Odpowiednio dobrany błonnik może ograniczać negatywne skutki ich działania, co wskazuje na możliwość zwiększenia bezpieczeństwa stosowania nanoform miedzi w żywieniu.

Słowa kluczowe: szczury, miedź, nanocząstki, immunologia, redoks

Summary

The aim of this research was to determine whether the immunological and oxidative–reductive response of the organism to copper nanoparticle (CuNPs) supplementation depends on the physiological properties of dietary fiber. It was hypothesized that the biological effects of CuNPs are dose-dependent and modulated by the type of dietary fiber, which, through its impact on the intestinal environment and microbiota, may regulate their bioavailability and metabolic effects.

The study was conducted on Wistar rats using a two-factor experimental design, including two levels of copper (6.5 and 13 mg/kg), two forms of copper ($CuCO_3$ and CuNPs), and four types of dietary fiber (cellulose, inulin, pectin, and psyllium). The effects of these factors on gastrointestinal function, oxidative–reductive status, copper bioavailability and distribution, liver metabolism, and immune response were evaluated.

The results demonstrated that CuNPs exhibit higher bioavailability compared to conventional inorganic forms, leading to altered tissue distribution and affecting the homeostasis of other minerals. At the same time, CuNPs were shown to induce oxidative stress and modulate immune responses in a dose-dependent manner.

It was found that the intestine plays a key role in regulating the organism's response to CuNPs. Interactions between nanoparticles and gut microbiota influence the production of short-chain fatty acids, the integrity of the intestinal barrier, and inflammatory processes, thereby determining systemic effects.

Dietary fiber was identified as a significant modulator of CuNPs' biological effects. Inulin exhibited protective effects at the recommended dose, whereas pectin and psyllium reduced oxidative stress and inflammatory responses under higher CuNPs intake. These differences result from the distinct physicochemical properties of dietary fiber.

In conclusion, the biological effects of CuNPs depend on the interaction between nanoparticle dose and dietary composition. Appropriately selected dietary fiber may mitigate the adverse effects of CuNPs, indicating its potential role in improving the safety of copper nanoparticle application in nutrition.

Keywords: rats, copper, nanoparticles, immunology, redox status

1. Wstęp

Postęp w dziedzinie nanotechnologii w ostatnich latach doprowadził do intensywnego rozwoju zastosowań nanocząstek w medycynie, farmacji, przemyśle spożywczym oraz żywieniu zwierząt. Szczególne zainteresowanie budzą nanocząstki metali, których właściwości fizykochemiczne tj. niewielki rozmiar, zwiększona powierzchnia właściwa oraz odmienna kinetyka reakcji biologicznych mogą istotnie zmieniać ich biodostępność, reaktywność oraz sposób oddziaływania na organizm (Frohlich i Frohlich, 2016; Cao, 2018). W kontekście żywienia nanoformy pierwiastków rozważane są jako alternatywa dla tradycyjnych soli nieorganicznych oraz form organicznych, jednak ich wpływ biologiczny pozostaje niejednoznaczny i silnie zależy od kontekstu dietetycznego oraz fizjologicznego organizmu (Frohlich i Frohlich, 2016; Ognik i in., 2020).

Miedź (Cu) należy do mikroelementów niezbędnych dla prawidłowego wzrostu, rozwoju oraz funkcjonowania organizmów żywych. Wchłanianie miedzi zachodzi w różnych odcinkach przewodu pokarmowego, a jej homeostaza regulowana jest przez złożony system krążenia jelitowo-wątrobowego, obejmujący wydzielanie z żółcią oraz ponowne wchłanianie (Turnlund i in., 1985). Szacuje się, że znaczna część miedzi docierającej do jelita cienkiego ulega ponownemu wydzieleniu do światła przewodu pokarmowego, co stanowi istotny mechanizm regulujący jej biodostępność. Dystrybucja miedzi w ustroju zachodzi głównie z udziałem ceruloplazminy i albuminy, a także innych białek wiążących, które uczestniczą w jej transporcie i magazynowaniu (Linder M., 2020).

Na poziomie komórkowym miedź pełni funkcję kofaktora licznych enzymów, w tym CuZn-SOD, oksydazy cytochromu c, oksydazy lizylowej, tyrozynazy oraz β-hydroksylazy dopaminy (Ognik i in., 2020; Nam i in., 2019). Enzymy te uczestniczą w procesach oddychania mitochondrialnego, syntezie tkanki łącznej, metabolizmie neuroprzekaźników oraz mechanizmach obrony antyoksydacyjnej (Nam i in., 2019). Jednocześnie miedź, jako metal przejściowy, może katalizować reakcje prowadzące do powstawania reaktywnych form tlenu (ROS), co nadaje jej działaniu charakter dualny - fizjologicznie niezbędny, lecz potencjalnie toksyczny przy nadmiernej kumulacji w komórkach (Bagheri i in., 2018). Mechanizmy zabezpieczające przed toksycznym działaniem miedzi obejmują jej wiązanie przez białka bogate w grupy tiolowe oraz udział wyspecjalizowanych białek transportowych, które kontrolują jej wewnątrzkomórkową dystrybucję i wbudowywanie w struktury enzymatyczne (Rosenzweig, 2002; Blindauer, 2015). Zaburzenie tych mechanizmów prowadzi do nasilenia procesów oksydacyjnych, peroksydacji lipidów, uszkodzeń DNA oraz

deregulacji ekspresji genów (Bagheri i in., 2018). Wykazano, że zarówno niedobór, jak i nadmiar miedzi mogą prowadzić do zaburzeń funkcjonowania układu odpornościowego, zmian neurodegeneracyjnych oraz nieprawidłowości metabolicznych (Nam i in., 2019).

W ostatniej dekadzie szczególną uwagę poświęcono nanocząsteczkom metali, w tym nanocząsteczkom miedzi (Cu-NP), które ze względu na swoje właściwości fizykochemiczne wykazują zwiększoną biodostępność oraz odmienny profil dystrybucji tkankowej w porównaniu z konwencjonalnymi formami miedzi (Cholewińska i in., 2018b; Ognik i in., 2020). Jednocześnie liczba badań analizujących wpływ nanomateriałów przyjmowanych drogą pokarmową na organizm, w tym na przewód pokarmowy i mikrobiotę jelitową, pozostaje ograniczona (Frohlich i Frohlich, 2016). Nanocząstki miedzi wykazują silne właściwości przeciwdrobnoustrojowe i znajdują zastosowanie w farmaceutykach oraz kosmetologii (Ermini i Voliani, 2021), a także coraz częściej rozważane są jako składnik suplementów diety (Baravkar i in., 2021).

Doniesienia literaturowe wskazują zarówno na ich potencjalne działanie korzystne tj. przeciwzapalne, przeciwcukrzycowe oraz kardioprotekcyjne (Sharma i in., 2018) jak i na możliwość wywoływania efektów toksycznych, w tym zmian histopatologicznych oraz zaburzeń równowagi redoks, zależnych od dawki i czasu ekspozycji (Kiyani i in., 2021). W modelach zwierzęcych wykazano m.in. poprawę wytrzymałości mechanicznej kości przy niskich dawkach Cu-NP (Tomaszewska i in., 2017), modulację funkcji naczyń krwionośnych (Majewski i in., 2017; 2019), a także wpływ na markery stresu oksydacyjnego (Majewski i in., 2020). Jednocześnie obserwowano zwiększoną akumulację miedzi w mózgu oraz zmiany morfologiczne w wątrobie (Cholewińska i in., 2018b), a także potencjalne implikacje neurobiologiczne związane ze zmianami poziomu β-amyloidu i aktywności acetylocholinesterazy (Cendrowska-Pinkosz i in., 2021).

Łącznie wyniki te wskazują na „dwoisty" charakter działania nanocząsteczek miedzi, obejmujący zarówno efekty korzystne, jak i niepożądane, co sugeruje konieczność uwzględnienia czynników modulujących ich działanie. Jednym z najważniejszych z nich wydaje się środowisko przewodu pokarmowego, w tym skład mikrobioty jelitowej oraz obecność składników diety.

Coraz więcej dowodów wskazuje, że nanocząstki mogą oddziaływać z mikroorganizmami jelitowymi, wpływając na ich liczebność oraz aktywność metaboliczną (Azam i in., 2012; Tang i in., 2021). Wykazano, że Cu-NP mogą obniżać produkcję krótkołańcuchowych kwasów tłuszczowych (SCFA) oraz aktywność enzymatyczną mikrobioty jelitowej, co może prowadzić do zaburzeń funkcjonowania bariery jelitowej oraz

modulacji odpowiedzi immunologicznej (Cholewińska i in., 2018a). SCFA odgrywają kluczową rolę w utrzymaniu integralności nabłonka jelitowego, regulacji procesów zapalnych oraz komunikacji jelitowo-ogólnoustrojowej.

Istotną rolę w kształtowaniu środowiska jelitowego odgrywa błonnik pokarmowy, którego właściwości fizykochemiczne takie jak fermentowalność, lepkość czy zdolność wiązania wody - determinują jego wpływ na mikrobiotę jelitową oraz biodostępność składników mineralnych (Baye i in., 2017; Turnlund i in., 1985). Wykazano, że różne frakcje błonnika mogą modulować pH treści jelitowej, produkcję SCFA oraz transformację kwasów żółciowych, co pośrednio wpływa na wchłanianie i metabolizm miedzi (Fotschki i in., 2017). Ponadto niektóre typy włókien mogą wpływać na bilans mineralny poprzez oddziaływanie na dostępność antagonistów mineralnych (Baye i in., 2017).

Pomimo rosnącej liczby badań dotyczących nanocząsteczek miedzi, większość z nich koncentruje się na pojedynczych aspektach ich działania, takich jak biodostępność, status oksydacyjno-redukcyjny czy zmiany histologiczne. Brakuje natomiast badań kompleksowych, integrujących jednocześnie wpływ Cu-NP na mikrobiotę jelitową, metabolizm, homeostazę mineralną, odpowiedź immunologiczną oraz status oksydoredukcyjny, z uwzględnieniem modulującej roli różnych typów błonnika pokarmowego.

2. Hipoteza badawcza i cel badań

Postawiono hipotezę, że biologiczne działanie nanocząsteczek miedzi (CuNPs) w organizmie zwierząt ma charakter zależny od dawki i jest istotnie modulowane przez rodzaj błonnika pokarmowego obecnego w diecie. Założono, że funkcjonalne frakcje błonnika, zwłaszcza fermentujące (inulina i pektyna), poprzez wpływ na mikrobiotę jelitową, produkcję krótkołańcuchowych kwasów tłuszczowych oraz integralność bariery jelitowej, mogą regulować biodostępność CuNPs, ograniczać stres oksydacyjny oraz modyfikować odpowiedź metaboliczną i immunologiczną organizmu. W konsekwencji przyjęto, że odpowiednio dobrana dieta może zwiększyć bezpieczeństwo stosowania nanoform miedzi.

Głównym celem badań było wyjaśnienie mechanizmów odpowiedzialnych za zależną od diety modulację efektów biologicznych CuNPs oraz określenie roli błonnika pokarmowego jako czynnika regulującego bezpieczeństwo i kierunek działania nanoform miedzi. W szczególności celem było określenie wpływu badanych czynników na: funkcjonowanie przewodu pokarmowego, w tym aktywność mikrobioty jelitowej, status oksydacyjno-redukcyjny organizmu, biodostępność i dystrybucję miedzi w tkankach, wybrane aspekty metabolizmu wątrobowego oraz odpowiedź immunologiczną i parametry hematologiczne.

3. Materiał i metody

3.1 Układ doświadczenia

Doświadczenie przeprowadzono na samcach szczurów Wistar (Cmdb:Wi), pochodzących z certyfikowanej hodowli laboratoryjnej (Instytut Rozrodu Zwierząt i Badań Żywności PAN, Olsztyn, Polska), które po okresie adaptacji losowo przydzielono do grup doświadczalnych. Zwierzęta utrzymywano przez 6 tygodni w standardowych warunkach laboratoryjnych (temperatura 21–22°C, wilgotność względna 50–60%, cykl świetlny 12 h światło/12 h ciemność) z nieograniczonym dostępem do wody i paszy.

Eksperyment zaprojektowano w układzie dwuczynnikowym obejmującym:

- **dwie dawki miedzi**: 6,5 mg/kg diety (dawka rekomendowana) oraz 13 mg/kg diety (dawka podwójna),
- **cztery typy błonnika pokarmowego**: celuloza (kontrola), inulina, pektyna oraz psyllium.

Doświadczenie obejmowało dwie grupy kontrolne otrzymujące $CuCO_3$ oraz osiem grup eksperymentalnych otrzymujących CuNPs w dwóch dawkach (6,5 i 13 mg/kg diety) w połączeniu z różnymi źródłami błonnika pokarmowego. Łącznie utworzono 10 grup doświadczalnych (n = 10 zwierząt w grupie).

Wszystkie procedury doświadczalne przeprowadzono zgodnie z obowiązującymi przepisami dotyczącymi ochrony zwierząt wykorzystywanych do celów naukowych (Dyrektywa 2010/63/UE). Zgoda na przeprowadzenie doświadczenia została wydana przez Lokalną Komisję Etyczną do spraw Doświadczeń na Zwierzętach w Olsztynie nr zgody 19/2021; Olsztyn, Polska.

Zwierzęta karmiono półoczyszczonymi dietami przygotowywanymi laboratoryjnie na bazie diety AIN-93G. Głównym źródłem białka była kazeina, tłuszczu - olej rzepakowy, a energii - skrobia kukurydziana. Celuloza (8% diety) stanowiła kontrolne źródło błonnika, natomiast w grupach eksperymentalnych zastępowano ją odpowiednio inuliną, pektyną lub psyllium (6% diety). W grupach kontrolnych miedź wprowadzano w postaci $CuCO_3$ poprzez dokładne wymieszanie z premiksem mineralnym. W dietach zawierających CuNPs (cząstki o wielkości 40-60 nm) nanocząstki zawieszano w oleju rzepakowym w celu zapewnienia równomiernej dyspersji i minimalizacji strat materiału. Po połączeniu wszystkich składników diety mieszano do uzyskania jednorodnej konsystencji, a przed podaniem paszy zwierzętom każdą partię ponownie homogenizowano.

3.2 Analizy laboratoryjne

Po 6-tygodniowym okresie żywienia zwierzęta poddawano 8-godzinnej głodówce (dostęp do wody był stały), a następnie znieczulano (ketamina i ksylazyna, podanie dootrzewnowe, odpowiednio 100 mg i 10 mg/kg). Krew pobierano z żyły głównej dolnej do probówek z EDTA oraz heparyną. Po zakończeniu procedury zwierzęta uśmiercano metodą dyslokacji kręgów szyjnych. Osocze uzyskiwano poprzez wirowanie próbek krwi (350x g, 10 min, 4°C) i przechowywano w temperaturze −80°C do momentu analiz. Pobierano również tkanki (jelito cienkie, jelito ślepe i jego treść, wątroba, mózg, nerki, serce, mięśnie, śledzionę, płuca, trzustkę, jądra) do analiz biochemicznych, molekularnych i histologicznych.

W publikacji Cholewińska i in. (2023) analizowano:

a) stężenia krótkołańcuchowych kwasów tłuszczowych (SCFA) w treści jelita ślepego metodą chromatografii gazowej (GC),
b) funkcjonowanie przewodu pokarmowego poprzez oznaczenie aktywności enzymatycznej mikrobioty jelitowej, w tym aktywności enzymów bakteryjnych jelita ślepego (m.in. β-glukozydazy, β-glukuronidazy oraz α- i β-galaktozydazy), metodami spektrofotometrycznymi,
c) pH treści jelitowej, masę i długość poszczególnych odcinków przewodu pokarmowego
d) budowę histologiczną jelita cienkiego z oceną zmian morfologicznych i integralności bariery jelitowej na preparatach barwionych hematoksyliną i eozyną (H&E) przy użyciu mikroskopii świetlnej,
e) ekspresję genów związanych z odpowiedzią zapalną i funkcjonowaniem bariery jelitowej, w tym NF-κB oraz TLR-4, z zastosowaniem ilościowej reakcji łańcuchowej polimerazy w czasie rzeczywistym (qPCR), jako gen referencyjny wykorzystano GAPDH. Całkowite RNA izolowano przy użyciu komercyjnych zestawów (Syngen Biotech, Polska), a jego stężenie i czystość oceniano spektrofotometrycznie. Syntezę cDNA przeprowadzono z wykorzystaniem zestawu NG dART RT Kit (EURx, Polska), natomiast analizę ekspresji genów wykonano metodą SYBR Green przy użyciu aparatu Real-Time PCR,
f) poziom globalnej metylacji DNA oraz aktywność enzymu naprawczego APE-1 (endonukleaza apurynowo/apirymidynowa 1) z zastosowaniem komercyjnych zestawów diagnostycznych i metod immunoenzymatycznych.

W publikacji Marzec i in. (2024) oznaczano:

a) oznaczenie parametrów biochemicznych związanych z funkcją wątroby, w tym aktywności aminotransferazy alaninowej (ALT) i aminotransferazy asparaginianowej (AST),
b) oznaczenie zawartości tłuszczu całkowitego, cholesterolu oraz triglicerydów w tkance wątrobowej metodami spektrofotometrycznymi,
c) ocenę histologiczną wątroby na preparatach barwionych hematoksyliną i eozyną (H&E) metodą mikroskopii świetlnej obejmującą ocenę stłuszczenia hepatocytów, nacieków komórek zapalnych oraz innych zmian morfologicznych,
d) analizę ekspresji genów związanych z metabolizmem lipidów i procesami zapalnymi, w tym SREBP-1c, PPAR-γ oraz COX-2, w tkance wątroby metodą ilościowej reakcji PCR w czasie rzeczywistym (qPCR) po wcześniejszej izolacji całkowitego RNA i syntezie cDNA.

W publikacji Marzec i in. (2025a) oceniano:

a) testy bilansowe obejmujące oznaczenie pobrania, wydalania z kałem i moczem, strawności oraz stopnia wykorzystania miedzi w organizmie,
b) zawartość miedzi w próbkach diety, wody, kału, moczu oraz w tkankach (wątroba, mózg, mięsień) po wcześniejszej mineralizacji oznaczano metodą spektrometrii mas z plazmą sprzężoną indukcyjnie (ICP-MS),
c) stężenia pierwiastków mineralnych w osoczu (Cu, Ca, Mg, P, Fe, Zn) metodą absorpcyjnej spektrometrii atomowej (FAAS).

W publikacji Marzec i in. (2025b) oznaczano:

a) aktywności enzymów antyoksydacyjnych (SOD, CAT), całkowitego statusu antyoksydacyjnego (TAS), poziomu zredukowanego glutationu (GSH),
b) markery stresu oksydacyjnego i uszkodzeń biomolekuł, takich jak MDA, pochodne karbonylowe białek (PC), 8-hydroksy-2'-deoksyguanozyna (8-OHdG), 3-nitrotyrozyna (3-NT) oraz ceruloplazmina (Cp).

Wykonano je metodami spektrofotometrycznymi oraz immunoenzymatycznymi z wykorzystaniem komercyjnych zestawów zgodnie z procedurami producenta.

W publikacji Marzec i in. (2026) oznaczano:

a) parametry hematologiczne: liczbę leukocytów, limfocytów, neutrofili, erytrocytów, stężenie hemoglobiny oraz hematokryt (WBC, LYM, NEU, RBC, HGB, HCT),
b) wskaźniki erytrocytarne: średnią objętość krwinki czerwonej (MCV), średnią masę hemoglobiny w krwince czerwonej (MCH), średnie stężenie hemoglobiny w krwince czerwonej (MCHC) oraz wskaźnik zróżnicowania objętości erytrocytów (RDWc),
c) wskaźniki płytkowe: liczbę płytek krwi (PLT), średnią objętość płytek krwi (MPV), wskaźnik zróżnicowania objętości płytek krwi (PDWc) oraz płytkokryt (PCT). Parametry oznaczano przy użyciu automatycznego analizatora hematologicznego ABACUS Jr VET (Diatron, Węgry),
d) stężenia immunoglobulin (IgA, IgG, IgM), cytokin prozapalnych interleukiny 2, interleukiny 6 i czynnika martwicy nowotworów alfa (IL-2, IL-6, TNF-α) oraz białka C-reaktywnego (CRP) metodą immunoenzymatyczną ELISA z wykorzystaniem komercyjnych zestawów zgodnie z instrukcją producenta.

3.3 Analizy statystyczne

Analizę statystyczną przeprowadzono z wykorzystaniem programu STATISTICA (StatSoft Inc., Tulsa, OK, USA). Do oceny wpływu badanych czynników zastosowano dwuczynnikową analizę wariancji (ANOVA), uwzględniając główne efekty poziomu CuNPs w diecie (D), rodzaju błonnika (F) oraz ich interakcję (D×F). W przypadku istotnych różnic między grupami stosowano test post hoc (Duncana). Różnice uznawano za statystycznie istotne przy poziomie istotności $P \leq 0,05$.

4. Omówienie wyników i dyskusja

Badania opublikowane w pracy **Marzec i in. (2025a)** wykazały, że zastąpienie $CuCO_3$ nanocząsteczkami miedzi (CuNPs) istotnie zmienia gospodarkę tym pierwiastkiem w organizmie szczurów. Stwierdzono statystycznie istotne zmniejszenie wydalania miedzi z organizmu oraz zwiększenie jej strawności i wykorzystania, co jednoznacznie potwierdza wyższą biodostępność CuNPs. Jednocześnie wykazano istotne zmniejszenie akumulacji miedzi w mózgu i mięśniach, co wskazuje na zmianę kierunku dystrybucji pierwiastka w organizmie. Ponadto odnotowano istotny wzrost stężeń Ca, P, Mg, Zn i Fe w osoczu, co sugeruje obecność interakcji pomiędzy mikroelementami oraz możliwą konkurencję o wspólne mechanizmy transportowe.

W warunkach podwyższonej podaży CuNPs dodatek błonnika pokarmowego (inulina, pektyna, psyllium) prowadził do istotnego zwiększenia wydalania miedzi, przy jednoczesnym zachowaniu jej efektywnego wykorzystania, co wskazuje na zdolność błonnika do regulacji biodostępności tego pierwiastka.

Dodatkowo wykazano, że zwiększenie dawki CuNPs prowadziło do wzrostu całkowitego pobrania miedzi, jednak przy jednoczesnym uruchomieniu mechanizmów kompensacyjnych związanych z jej wydalaniem. Może to wskazywać na próbę utrzymania homeostazy pierwiastka przez organizm w warunkach zwiększonej biodostępności nanocząsteczek. Zaobserwowane zmiany w dystrybucji miedzi sugerują również, że CuNPs mogą wykazywać odmienne powinowactwo tkankowe niż tradycyjne sole nieorganiczne. Szczególnie istotne wydaje się ograniczenie akumulacji miedzi w mózgu, ponieważ nadmierne odkładanie tego pierwiastka w tkance nerwowej jest wiązane z nasileniem procesów neurodegeneracyjnych i stresem oksydacyjnym.

Uzyskane wyniki wskazują, że nanocząstki miedzi wykazują odmienny profil metaboliczny niż tradycyjne formy nieorganiczne. Zmniejszona akumulacja miedzi w mózgu może mieć znaczenie ochronne w kontekście potencjalnej neurotoksyczności tego pierwiastka, natomiast wzrost stężeń innych mikroelementów sugeruje występowanie złożonych interakcji pomiędzy szlakami transportu i metabolizmu metali. Jednocześnie obserwowany wpływ błonnika pokarmowego wskazuje, że skład diety może istotnie modulować losy ustrojowe CuNPs, ograniczając ryzyko zaburzeń homeostazy mineralnej. W literaturze podkreśla się, że zwiększona biodostępność CuNPs wynika przede wszystkim z ich niewielkiego rozmiaru, dużej powierzchni aktywnej oraz zdolności do przenikania przez enterocyty drogą endocytozy i pinocytozy (Lee i in., 2016; Ognik i in., 2016).

Dodatkowo część CuNPs może ulegać dysocjacji do jonów Cu^{2+} już w kwaśnym środowisku żołądka, co umożliwia ich wchłanianie z udziałem transporterów DMT1 i CTR1, podobnie jak tradycyjnych form nieorganicznych miedzi.

Zgodnie z obserwacjami Wijmenga i Klomp (2004) oraz Coudray i in. (1997), zwiększone wydalanie miedzi może stanowić jeden z podstawowych mechanizmów utrzymania homeostazy tego pierwiastka w warunkach jego nadmiernej biodostępności. Zaobserwowane zmiany stężeń Fe, Zn i Mg mogą wynikać z konkurencji pomiędzy jonami metali dwuwartościowych o wspólne transportery błonowe, szczególnie DMT1, którego aktywność obejmuje nie tylko miedź, ale również żelazo i cynk (Kim i in., 2008, Wapnir, 1998). W literaturze podkreśla się również, że zaburzenia homeostazy miedzi mogą wtórnie wpływać na metabolizm innych mikroelementów poprzez modulację ekspresji transporterów jelitowych i białek wiążących metale. Według Baye i in. (2017) oraz Wapnir (1998), skład diety i obecność błonnika pokarmowego mogą dodatkowo modulować biodostępność mikroelementów poprzez wpływ na pH treści jelitowej oraz tworzenie kompleksów z jonami metali.

Badania opublikowane w pracy **Cholewińska i in. (2023)** wykazały, że CuNPs w sposób istotny wpływają na parametry integralności jelita cienkiego. Stwierdzono statystycznie istotne zmniejszenie poziomu kwasu mlekowego oraz markerów apoptozy, co może wskazywać na ograniczenie degradacji komórek nabłonka jelitowego i zwiększenie ich przeżywalności. Jednocześnie zaobserwowano istotne zwiększenie uszkodzeń DNA, świadczące o nasileniu stresu oksydacyjnego i potencjalnym działaniu genotoksycznym CuNPs. Choć ograniczenie apoptozy może być interpretowane jako przejaw adaptacji komórek do działania czynnika stresowego, współwystępowanie uszkodzeń materiału genetycznego sugeruje, że efekt ten nie musi być jednoznacznie korzystny biologicznie.

W badaniach wykazano również, że zastosowanie funkcjonalnych frakcji błonnika wpływało na parametry środowiska jelitowego, w tym aktywność mikrobioty oraz produkcję SCFA, które odgrywają istotną rolę w utrzymaniu integralności bariery jelitowej. Może to częściowo tłumaczyć obserwowane działanie ochronne inuliny i pektyny względem zmian indukowanych przez CuNPs.

Zaobserwowane zwiększenie uszkodzeń DNA przy jednoczesnym ograniczeniu apoptozy może również, jak wspomniano wcześniej, wskazywać na rozwój mechanizmów adaptacyjnych komórek jelitowych wobec przewlekłej ekspozycji na CuNPs. Taki efekt może jednak prowadzić do utrzymywania się komórek z uszkodzonym materiałem

genetycznym, co podkreśla konieczność ostrożnej oceny bezpieczeństwa długotrwałego stosowania nanocząsteczek miedzi w żywieniu.

Wykazano również, że inulina istotnie wspiera mechanizmy naprawy DNA, natomiast pektyna ogranicza procesy zapalne i apoptozę enterocytów.

Uzyskane wyniki wskazują, że działanie CuNPs w jelicie ma charakter złożony i dwukierunkowy. Z jednej strony obserwuje się efekty adaptacyjne, związane z ograniczeniem apoptozy, z drugiej wyraźne działanie prooksydacyjne wynikające prawdopodobnie z lokalnego uwalniania jonów miedzi i indukcji reakcji wolnorodnikowych. Inulina, jako błonnik fermentujący, może działać ochronnie poprzez modulację aktywności mikrobioty jelitowej i zwiększenie produkcji metabolitów wspierających integralność bariery jelitowej. Z kolei pektyna, poprzez zwiększenie lepkości treści jelitowej, może ograniczać kontakt CuNPs z powierzchnią enterocytów. Jelito należy zatem uznać za kluczowy punkt regulacyjny odpowiedzi organizmu na nanocząstki miedzi, determinujący dalsze efekty ogólnoustrojowe.

Wyniki te są zgodne z doniesieniami wskazującymi, że CuNPs mogą indukować stres oksydacyjny poprzez generowanie reaktywnych form tlenu w wyniku reakcji Fentona i Haber–Weissa (Bost i in., 2016; Ognik i in., 2020; Adams i in., 2018). Powstające ROS mogą prowadzić do uszkodzeń lipidów, białek oraz materiału genetycznego enterocytów, szczególnie w tkankach bezpośrednio eksponowanych na kontakt z nanocząstkami.

Strauch i in. (2017) opisali tzw. mechanizm „Trojan horse", zgodnie z którym CuNPs po endocytozie ulegają degradacji w lizosomach, prowadząc do wewnątrzkomórkowego przeciążenia jonami Cu^{2+} i aktywacji szlaków związanych z uszkodzeniem DNA oraz stresem oksydacyjnym.

Maślan stanowi podstawowe źródło energii dla kolonocytów oraz uczestniczy w regulacji ekspresji białek połączeń ścisłych, ograniczając przepuszczalność bariery jelitowej. W piśmiennictwie wskazuje się, że błonnik rozpuszczalny, szczególnie pektyna i psyllium, może ograniczać biodostępność metali poprzez zwiększanie lepkości treści jelitowej oraz tworzenie kompleksów z jonami dwuwartościowymi (Krzysik i in., 2009). Dostępne dane wskazują, że fermentujące frakcje błonnika, szczególnie inulina, zwiększają produkcję krótkołańcuchowych kwasów tłuszczowych (SCFA), które odgrywają istotną rolę w utrzymaniu integralności bariery jelitowej oraz regulacji odpowiedzi immunologicznej (Lee i in., 2015;2021; Baye i in., 2017).

Badania opublikowane w pracy **Marzec i in. (2024)** wykazały, że wpływ CuNPs na metabolizm wątrobowy jest silnie zależny od dawki oraz rodzaju błonnika pokarmowego.

Stwierdzono statystycznie istotny wzrost akumulacji lipidów, cholesterolu i triglicerydów w wątrobie przy wyższej dawce CuNPs, czemu towarzyszyła aktywacja procesów zapalnych oraz zmiany histopatologiczne, w tym nacieki komórek zapalnych i cechy stłuszczenia wątroby.

Jednocześnie wykazano, że zastosowanie błonnika, szczególnie psyllium i inuliny, istotnie obniża poziomy lipidów, ogranicza zmiany histologiczne oraz zmniejsza ekspresję genów związanych z lipogenezą i stanem zapalnym, takich jak SREBP-1c, PPAR-γ oraz COX-2.

Wykazano ponadto, że zastosowanie psyllium wywierało najsilniejszy efekt ochronny względem zmian metabolicznych zachodzących w wątrobie. Obserwowano ograniczenie nacieków komórek zapalnych oraz zmniejszenie stopnia stłuszczenia hepatocytów, co znajdowało odzwierciedlenie zarówno w analizie histopatologicznej, jak i ekspresji genów związanych z lipogenezą. Wyniki te sugerują, że błonnik o właściwościach pęczniejących może skutecznie ograniczać nadmierne wchłanianie CuNPs lub modulować wtórne skutki metaboliczne ich działania.

Na uwagę zasługuje również fakt, że zaburzenia metabolizmu lipidów obserwowane przy wyższej podaży CuNPs były związane ze zwiększoną ekspresją genów odpowiedzialnych za syntezę i magazynowanie lipidów. Może to wskazywać, że nadmierna biodostępność miedzi wpływa na szlaki regulujące gospodarkę energetyczną hepatocytów, prowadząc do rozwoju zmian przypominających metabolicznie uwarunkowaną stłuszczeniową chorobę wątroby (MASLD).

Uzyskane wyniki wskazują, że zwiększona biodostępność CuNPs może prowadzić do zaburzeń metabolizmu lipidów i inicjacji procesów o charakterze stłuszczeniowym oraz zapalnym w wątrobie. Mechanizm ten może być związany zarówno z bezpośrednim działaniem CuNPs, jak i wtórnymi efektami wynikającymi ze zmian zachodzących w jelicie, w tym zaburzeń bariery jelitowej i zwiększonego napływu czynników prozapalnych. Obserwowane działanie ochronne błonnika wskazuje, że modulacja procesów zachodzących w przewodzie pokarmowym może skutecznie ograniczać wtórne skutki metaboliczne zwiększonej ekspozycji na CuNPs w obrębie osi jelito-wątroba.

W literaturze podkreśla się, że nadmierna ekspozycja na CuNPs może prowadzić do zaburzeń metabolizmu lipidów, nasilenia procesów zapalnych oraz rozwoju stłuszczenia wątroby poprzez aktywację mechanizmów oksydacyjnych i prozapalnych (Liu i in., 2022). Szczególnie istotną rolę przypisuje się tutaj aktywacji szlaków związanych z SREBP-1c, PPAR-γ oraz COX-2, regulujących lipogenezę i odpowiedź zapalną hepatocytów.

Badania wskazują również, że psyllium oraz inulina mogą ograniczać akumulację lipidów w wątrobie poprzez wpływ na metabolizm kwasów żółciowych, mikrobiotę jelitową oraz zmniejszenie dostępności wolnych jonów miedzi w świetle przewodu pokarmowego.

Badania opublikowane w pracy **Marzec i in. (2025b)** wykazały, że wpływ CuNPs na status oksydacyjno-redukcyjny organizmu jest determinowany przede wszystkim przez interakcję pomiędzy dawką a rodzajem błonnika pokarmowego. Stwierdzono statystycznie istotne interakcje dla poziomu MDA oraz aktywności enzymów antyoksydacyjnych (SOD, CAT) w licznych tkankach.

Wykazane zależności miały wyraźnie tkankowo swoisty charakter, co sugeruje, że poszczególne narządy różnią się wrażliwością na działanie CuNPs oraz zdolnością do aktywacji mechanizmów antyoksydacyjnych. Szczególnie istotne zmiany obserwowano w wątrobie, jelicie oraz tkankach bezpośrednio związanych z metabolizmem i dystrybucją miedzi. Może to wynikać z różnic w lokalnym nasileniu reakcji Fentona oraz odmiennym poziomie endogennych systemów antyoksydacyjnych.

Zaobserwowane działanie ochronne inuliny, pektyny i psyllium może być związane nie tylko z ograniczeniem biodostępności jonów miedzi, ale również z pośrednim wpływem na mikrobiotę jelitową oraz produkcję metabolitów o działaniu przeciwzapalnym i antyoksydacyjnym. Potwierdza to istotną rolę osi jelito–mikrobiota–status oksydoredukcyjny w odpowiedzi organizmu na nanocząstki metali.

Wykazano, że zastosowanie inuliny przy dawce rekomendowanej prowadziło do poprawy parametrów antyoksydacyjnych, w tym ograniczenia peroksydacji lipidów oraz stabilizacji równowagi oksydoredukcyjnej. Natomiast przy podwyższonej podaży CuNPs działanie ochronne wykazywały głównie pektyna i psyllium, które ograniczały nasilenie stresu oksydacyjnego.

Uzyskane wyniki wskazują, że CuNPs mogą indukować stres oksydacyjny poprzez generację reaktywnych form tlenu, jednak efekt ten jest silnie zależny od kontekstu dietetycznego. Błonnik pokarmowy pełni kluczową rolę w modulowaniu odpowiedzi oksydoredukcyjnej, a jego działanie ma charakter zależny od dawki CuNPs oraz właściwości fizykochemicznych danej frakcji błonnika. Wskazuje to na konieczność uwzględnienia interakcji dieta–nanocząstki w ocenie bezpieczeństwa ich stosowania.

Wyniki te są zgodne z doniesieniami wskazującymi, że CuNPs mogą wykazywać zarówno działanie prooksydacyjne, jak i adaptacyjne, zależnie od dawki oraz rodzaju tkanki (Ognik i in., 2020). Z jednej strony nanocząstki miedzi nasilają generowanie ROS, z drugiej

mogą aktywować mechanizmy kompensacyjne związane z odpowiedzią antyoksydacyjną organizmu.

Badania opublikowane w pracy **Marzec i in. (2026)** wykazały, że CuNPs istotnie modulują funkcjonowanie układu immunologicznego. Stwierdzono statystycznie istotny wzrost poziomów cytokin prozapalnych (IL-6, TNF-α), zmiany w liczbie leukocytów oraz poziomach immunoglobulin, co wskazuje na aktywację odpowiedzi zapalnej.

Jednocześnie wykazano, że zastosowanie inuliny i psyllium istotnie obniża poziomy IL-6, TNF-α oraz IgM, co świadczy o ich działaniu immunomodulującym i przeciwzapalnym.

Uzyskane wyniki sugerują, że zwiększona biodostępność CuNPs może prowadzić do aktywacji układu odpornościowego poprzez mechanizmy związane ze stresem oksydacyjnym oraz zaburzeniami środowiska jelitowego. Wzrost poziomu cytokin prozapalnych może być wynikiem aktywacji szlaków sygnałowych związanych z odpowiedzią na stres oksydacyjny oraz obecność jonów miedzi w komórkach. Jednocześnie błonnik pokarmowy może łagodzić te efekty poprzez poprawę integralności bariery jelitowej, modulację mikrobioty oraz ograniczenie procesów zapalnych. Wskazuje to na ścisłe powiązanie pomiędzy statusem oksydoredukcyjnym, funkcją jelita oraz odpowiedzią immunologiczną organizmu. Dodatkowo obserwowane zmiany mogą być związane z wpływem CuNPs na skład i aktywność mikrobioty jelitowej, która odgrywa kluczową rolę w dojrzewaniu oraz regulacji odpowiedzi immunologicznej gospodarza. Zaburzenia homeostazy mikrobiologicznej indukowane przez nanocząstki mogą prowadzić do aktywacji mechanizmów prozapalnych oraz zwiększonej produkcji cytokin zapalnych, w tym IL-6 i TNF-α (Broom i Kogut, 2018; Schokker i in., 2017). Jednocześnie fermentujące frakcje błonnika, szczególnie inulina, poprzez zwiększenie produkcji krótkołańcuchowych kwasów tłuszczowych (SCFA), mogą wspierać integralność bariery jelitowej i ograniczać nadmierną aktywację układu odpornościowego (Rubio, 2019; Rodrigues i in., 2021).

Uzyskane w całym cyklu wyniki wskazują, że biologiczne działanie nanocząsteczek miedzi ma charakter wieloetapowy i jest determinowane zarówno przez ich zwiększoną biodostępność, jak i kontekst dietetyczny. Kluczowym elementem regulującym odpowiedź organizmu jest jelito, które jako pierwsze miejsce kontaktu z CuNPs decyduje o ich wchłanianiu, wpływie na integralność bariery jelitowej oraz środowisko mikrobiologiczne. Zmiany zachodzące na poziomie jelita determinują następnie funkcjonowanie wątroby poprzez modulację dostępności miedzi, integralności bariery jelitowej oraz napływu sygnałów metabolicznych i zapalnych. Następstwem tych procesów są obserwowane zmiany statusu oksydacyjno-redukcyjnego oraz odpowiedzi immunologicznej organizmu.

Równolegle CuNPs modulują status oksydacyjno-redukcyjny oraz odpowiedź immunologiczną organizmu, przy czym efekty te mają charakter zależny od dawki i mogą przechodzić od adaptacyjnych do prooksydacyjnych i prozapalnych. Istotnym czynnikiem modyfikującym te procesy jest błonnik pokarmowy, którego różne frakcje wykazują odmienne mechanizmy działania – od regulacji biodostępności miedzi, przez stabilizację środowiska jelitowego, po ograniczenie stresu oksydacyjnego i odpowiedzi zapalnej. Wskazuje to, że ostateczny efekt działania CuNPs nie jest jednoznaczny, lecz wynika z interakcji pomiędzy dawką nanocząsteczek a składem diety, co podkreśla znaczenie żywieniowej modulacji ich bezpieczeństwa i efektywności biologicznej.

Podsumowując należy podkreślić, że choć badania przeprowadzono na szczurach laboratoryjnych jako zwierzętach modelowych, uzyskane wyniki **mogą mieć istotne znaczenie aplikacyjne dla nauk zootechnicznych**. Szczur jest powszechnie wykorzystywanym modelem w badaniach nad żywieniem, metabolizmem składników pokarmowych, funkcjonowaniem mikrobioty jelitowej oraz mechanizmami odpowiedzi immunologicznej i antyoksydacyjnej. Poznanie zależności pomiędzy formą podawanego mikroelementu, zastosowanym błonnikiem, środowiskiem jelitowym a reakcją organizmu może stanowić podstawę do opracowania bezpieczniejszych i bardziej efektywnych strategii suplementacji mineralnej u zwierząt gospodarskich. Wyniki niniejszych badań mogą zatem przyczynić się do lepszego zrozumienia biologicznych skutków stosowania nanocząsteczek miedzi w żywieniu zwierząt oraz wspierać rozwój nowoczesnych rozwiązań żywieniowych w produkcji zwierzęcej.

5. Wnioski

1) Nanocząstki miedzi (CuNPs), dzięki niewielkim rozmiarom i dużej powierzchni właściwej, wykazują wyższą biodostępność niż tradycyjne formy nieorganiczne, co sprzyja ich efektywniejszemu wchłanianiu oraz kumulacji w tkankach. Mechanizm ten może być związany m.in. z ułatwioną internalizacją komórkową i zwiększonym uwalnianiem jonów Cu^{2+}. W konsekwencji dochodzi do zaburzeń homeostazy miedzi i interakcji z innymi pierwiastkami, a przy wyższych dawkach do nasilenia stresu oksydacyjnego.
2) Jelito odgrywa kluczową rolę w regulacji odpowiedzi organizmu na CuNPs, wpływając na ich biodostępność oraz efekt biologiczny. Interakcja CuNPs z mikrobiotą jelitową prowadzi do zmian jej aktywności metabolicznej, w tym produkcji SCFA, co może wpływać na integralność bariery jelitowej i nasilenie procesów zapalnych. Mechanizmy te, m.in. poprzez aktywację szlaków TLR-4/NF-κB, mogą oddziaływać ogólnoustrojowo, w tym na funkcjonowanie wątroby oraz status oksydacyjno-redukcyjny organizmu.
3) Rodzaj błonnika pokarmowego istotnie moduluje biologiczne efekty działania CuNPs. Frakcje fermentujące, szczególnie inulina, poprzez zwiększenie produkcji SCFA i poprawę środowiska jelitowego, wykazują działanie ochronne, ograniczając stres oksydacyjny i odpowiedź zapalną. Z kolei inne typy błonnika mogą wpływać na biodostępność miedzi poprzez jej wiązanie lub zmianę warunków w świetle jelita. Wskazuje to na kluczową rolę diety w regulacji bezpieczeństwa i efektów metabolicznych nanocząstek miedzi.

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nutrients

Article

The Effect of Copper Nanoparticles and a Different Source of Dietary Fibre in the Diet on the Integrity of the Small Intestine in the Rat

Ewelina Cholewińska [1,*], Aleksandra Marzec [1], Przemysław Sołek [1], Bartosz Fotschki [2], Piotr Listos [3], Katarzyna Ognik [1] and Jerzy Juśkiewicz [2,*]

[1] Department of Biochemistry and Toxicology, Faculty of Animal Sciences and Bioeconomy, University of Life Sciences, 20-950 Lublin, Poland; aleksandra.marzec@up.lublin.pl (A.M.); pp.solek@gmail.com (P.S.)
[2] Division of Food Science, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, 10-748 Olsztyn, Poland
[3] Department of Pathomorphology and Forensic Medicine, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, 20-612 Lublin, Poland
* Correspondence: ewelina.cholewinska@up.lublin.pl (E.C.); j.juskiewicz@pan.olsztyn.pl (J.J.)

Abstract: The aim of the study was to verify the hypothesis regarding the effect of 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 selected biological parameters of intestinal integrity in rats. Rats were randomly divided into 10 groups. The first two groups were fed a control diet that contained cellulose, and a mineral mixture with standard or enhanced content of $CuCO_3$. 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 and small intestine samples were collected for further analysis. Replacing $CuCO_3$ by CuNPs in the diet positively reduced the level of lactic acid and apoptosis markers in the small intestine; however, it also resulted in the intensification of DNA oxidation. The most beneficial effect on DNA repair mechanisms is related to inulin, while pectin has the greatest ability to inhibit inflammatory processes that induce the apoptotic death of cells in the small intestine. Our results suggest that dietary fibre supplementation protects the small intestine against potentially harmful, oxidative effects of CuNPs by intensifying the intestinal barrier.

Keywords: apoptosis; copper nanoparticles; dietary fibre; integrity of small intestine; inulin; pectin; psyllium; rat

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1. Introduction

Copper (Cu) is a trace element involved in many physiological processes in the living organism. Importantly, Cu is a cofactor of numerous metabolic enzymes, engaged in the energy production in the respiratory chain, free radical deactivation, the maintenance of connective tissue and the proper function or modulation of nerve conduction as a result of the conversion of dopamine to norepinephrine [1–7]. This element also stimulates the circulatory system by iron metabolism, as well as the coagulation process and blood pressure regulation [2,6,7]. In order to ensure the proper course of the above-mentioned physiological processes in the animal's body, it is therefore very important to provide a constant source of Cu in the diet. For this purpose, the diet is most often supplemented with a mineral mixture containing Cu at a level consistent with dietary recommendations, most often in the form of inorganic salt like $CuCO_3$ [8–10]. However, in recent years, researchers have been giving Cu nanoparticles (CuNPs) more attention due to the specific properties determining their higher bioavailability [8–10]. We previously showed that CuNPs included in the diet of rats are better absorbed from the gastrointestinal tract than

standard $CuCO_3$ [11], but most of all, they stimulate the immune system [12] and reduce the level of DNA methylation or oxidative damage to proteins and DNA [13]. Nevertheless, others indicated that the application of CuNPs in animal nutrition, apart from the benefits, may carry a significant risk due to the potential toxicity of CuNPs [11,13–17]. Our results are partly consistent with these reports, as we proved an increase in Cu accumulation in the brain, kidneys and lungs, as well as an intensification of lipid peroxidation or impairment of antioxidant defence [18]. Due to such a broad physiological effect of CuNPs in the body, indicating positive and negative effects, it may be important to regulate the area of their reactivity.

Dietary fibre is one of the most important components of the diet, apart from nutrients and microelements. It is defined as parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the small intestine of humans and animals, with the participation of endogenous enzymes, and are completely or partially fermented in the large intestine [19]. Dietary fibre is usually divided into soluble and insoluble fibre. Soluble fibre includes fibre which can absorb water and thus significantly increase its volume. This group includes, above all, viscous forming a gel in contact with water pectin and sticky, swelling psyllium, but also non-viscous inulin of significant prebiotic importance. The best-known example of insoluble fibre is cellulose, which does not show a significant tendency to bind water in the digestive tract [20]. Reports indicate that a diet rich in dietary fibre supports the gut microbiome and reduces the risk of cardiovascular diseases and colorectal cancer development [21]. The data also suggest that inclusion of dietary fibre in the diet may modify the processes of mineral absorption in the small intestine [22–26]. Moreover, the absorption of micronutrients and their impact on the intestinal tissue largely depends on the type and amount of fibre in the diet, and also minerals homeostasis in the body [27]. It has been reported that manipulation of the fibre content of the diet may have an indirect effect on Cu bioavailability by altering the bioavailability of mineral antagonists [28]. The effect of vegetable fibres on copper absorption is varied. It has been reported that dietary α-cellulose did not reduce copper absorption [29]; hence, we assumed that our choice of α-cellulose as control fibre has been justified. Different dietary fibres substantially effect the small and large intestinal luminal pH values, e.g., through the enhancement of water binding or increased viscosity, as well as the stimulation of microbiota in SCFA production. The increase in intestinal pH affects copper absorbability, presumably because of a diminished concentration of free copper. There is evidence that the fraction of Cu tightly bound to bile remains unabsorbable during its passage through the gastrointestinal tract. Some studies have shown that hemicellulose induced a negative copper balance in adolescent human males, though pectin and intact cellulose were inactive. Other refined fibres and gums, such as locust bean and karaya gums, as well as carboxymethylcellulose, have been shown to be either without effect or beneficial to the trace element balance, including copper [30].

Therefore, we assumed that the dietary combination of CuNPs with different types of fibre—neutral cellulose (control), prebiotic inulin, sticky pectin or swelling psyllium—would affect the physiological intestinal response, thereby regulating the metabolic effect of CuNPs in the body. The aim of the study was to verify the hypothesis regarding the effect of recommended Cu/kg concentration (6.5 mg) or the two times higher (13.0 mg) level of CuNPs in the diet, in combination with different types of dietary fibre (i.e., cellulose, pectin, inulin or psyllium) on selected biological parameters of intestinal integrity and histology of the small intestine in rats.

2. Materials and Methods

2.1. Chemicals and Dietary Fibre

Copper nanoparticles (99.9% purity powder, 40–60 nm size, 12 m^2/g specific surface area (SSA), spherical morphology, 0.19 g/cm^3 bulk density, 8.9 g/cm^3 true density) were obtained from Sky Spring Nanomaterials Inc. (Houston, TX, USA). CuNPs with the same properties were also used in our previous studies [11–13,18,31]. α-Cellulose was used as a

control dietary fibre source (Sigma, Poznań, Poland). The following experimental dietary fibre sources were used: pectin (PectinE 440(I), Brouwland, Beverlo, Belgium), inulin (FrutafitTex, Sensus, The Netherlands) and psyllium (Psyllim husk powder, NaturaleBio, Rome, Italy).

2.2. Experimental Protocol

Healthy outbred male Wistar rats 6-weeks old (Cmdb:Wi) were fed a standard semi-purified rat diet supplemented with two CuNPs doses (recommended and two times higher; 6.5 and 13 mg/kg diet, respectively) and combined with different types of dietary fibre. The control diet contained a mineral mixture with standard and enhanced content of $CuCO_3$ (6.5 and 13 mg/kg diet). In the diets with Cu-NP the mineral mixture was deprived of $CuCO_3$ and the copper nanoparticles were added to the diet, along with rapeseed oil (as an emulsion) for operator safety. The control dietary fibre, α-cellulose, was added at 8% of the diet, while the experimental fibre preparations (inulin with prebiotic effect, psyllium with bulk effect, pectin with viscous effect) were added at 6% of the diet at the expense of cellulose preparation (Table 1). The experimental protocol consisted of 10 groups, n = 10 per group. All animal care and experimental schema were in accordance with the Polish legislation acts concerning animal experimentation and ethical practice according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, Directive 2010/63/EU [32]. It was approved by the Local Ethics Committee for Animal Experiments in Olsztyn Local Animal Care and Use Committee (Approval No. 19/2021; Olsztyn, Poland). The study was carried out in compliance with the ARRIVE guidelines. Every effort was made to minimise the suffering of the animals used in the experiment.

Table 1. The 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
Cu-NPs	0	0	6.5	13	6.5	13	6.5	13	6.5	13

[1] Casein preparation: crude protein 89.7%, crude fat 0.3%, ash 2.0% and water 8.0%. [2] α-Cellulose (SIGMA, Poznan, Poland), main source of dietary fibre. [3] AIN-93G-VM [33], g/kg mix: 3.0 nicotinic acid, 1.6 Ca pantothenate, 0.7 pyridoxine-HCl, 0.6 thiamin-HCl, 0.6 riboflavin, 0.2 folic acid, 0.02 biotin, 2.5 vitamin B-12 (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 D-3 (cholecalciferol, 400,000 IU g^{-1}), 0.075 vitamin K-1 (phylloquinone), 974.655 powdered sucrose. [4] In the experimental treatments with CuNPs, the MX was deprived of $CuCO_3$ and, in order to keep the operator safe while preparing the experimental diets, the CuNPs preparation was added as an emulsion, along with dietary rapeseed oil. This procedure was successfully applied in the study authors' previous experiments. [5] Maize starch preparation: crude protein 0.6%, crude fat 0.9%, ash 0.2%, total dietary fibre 0% and water 8.8%.; groups CN and CNH were fed diets with a supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 8% of cellulose dietary fibre source; groups PN and PNH were fed diets with a supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of pectin dietary fibre source; groups JN and JNH were fed diets with a supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of inulin dietary fibre source; groups SN and SNH were fed diets with a supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of psyllium dietary fibre source.

2.3. Sample Collection and Analyses

Body weight (BW) was monitored at the start and end of the study for each animal, while the diet consumption was checked daily. Before the termination of the study, the rats were starved for 8 h but had free access to water. On the last day, the animals were subjected to body fat and lean tissue content analysis with the aid of the time-domain nuclear magnetic resonance (NMR) protocol (Minispec LF90II Bruker, Bremen, Germany). The principle of the analysis is that the tissue contrast is high between fat and muscle based on relative relaxation times. Next, the rats were anaesthetised i.p. with ketamine and xylazine 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 heparinised tubes, and finally the rats were euthanised by cervical dislocation. After that, the small intestine, heart, kidneys and spleen were dissected and weighed. Samples of the small intestine were preserved for histopathological examination, and intestinal tissue homogenates were prepared and stored at −80 °C until analysis. Blood plasma was prepared by solidification and low-speed centrifugation (350× *g*, 10 min, 4 °C). Plasma samples were kept frozen at −80 °C until assay.

The pH values in the ileal digesta were measured with the aid of a pH/ion metre (model 301, Hanna Instruments, Vila do Conde, Portugal), while the dry matter (DM) concentration in the digesta was analysed by sample drying at 105 °C to a constant weight. The apparent digesta viscosity was measured in the supernatant fraction using the cone/plate viscometer (model LVDV II+, Brookfield Engineering Laboratories, Stoughton, MA, USA).

In the blood plasma, the level of selected indicators of intestinal integrity—lactic acid (LA) and diamine oxidase (DAO)—was determined using a commercial measurement enzyme-linked immunosorbent assay (ELISA) kit, following the protocol provided by the manufacturer (Shanghai Qayee Biotechnology Co. Ltd., Shanghai, China). Absorbances were measured at 450 nm via ELISA reader (Sunrise^{TM}, Tecan Group Ltd., Männedorf, Switzerland).

In homogenates prepared from the small intestine, the level of 8-hydroxydeoxyguanosine (8-OHdG), apurinic/apyrimidinic endonuclease 1 (APE-1), 8-oxoguanine DNA glycosylase (OGG1), lactic acid (LA), diamine oxidase (DAO) and the level of caspase 3 and caspase 8 were determined. These parameters were determined using a commercial measurement enzyme-linked immunosorbent assay (ELISA) kit, following the protocol provided by the manufacturer (Shanghai Qayee Biotechnology Co., Ltd., Shanghai, China). Absorbances were measured at 450 nm via ELISA reader (Sunrise^{TM}, Tecan Group Ltd., Männedorf, Switzerland).

2.4. RNA Extraction and Quantitative Real-Time PCR

RNA from the small intestine was extracted using Trizol Reagent (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's protocol. The isolated RNA yield was estimated spectrophotometrically (UV-VIS spectrophotometer Nabi, MicroDigital Co. Ltd., Gyeonggi, Republic of Korea), with integrity assessed electrophoretically by separation on 0.8% agarose gel. For complementary cDNA synthesis, 1 µg of RNA was reverse transcribed using the NG dART RT kit (EURX Ltd., Gdańsk, Poland), according to the manufacturer's instructions. Specific primers for evaluation of genes expression—zonula occludens-1 (*ZO-1*), occludin (*OCLN*), trefoil factor 2 (*TFF2*) and 8-oxoguanine glycosylase (*OGG1*)—were designed using Primer 3 software (Whitehead Institute, Cambridge, MA, USA) and synthesised by Genomed (Warsaw, Poland). The sequences are shown in Table 2.

Real-time PCR was performed on Quantabio thermocycler (VWR International LLC, Radnor, PA, USA) and using SG qPCR Master Mix (2×) (EURX Ltd., Gdańsk, Poland) according to the following protocol: one cycle at 95 °C for 10 min (initial denaturation), followed by a PCR including 35 cycles at 95 °C for 20 s (denaturation), 59–62 °C for 20 s (annealing) and 72 °C for 30 s (elongation). A melting curve analysis was performed over 50–72 °C at 0.3 °C/s intervals. Negative controls without the cDNA template were provided. Real-time PCR was performed in duplicate. Normalised gene expression was calculated

using the $2^{-\Delta Ct}$ method. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β-actin (*ACTB*) genes were selected as endogenous controls to normalise gene expression.

Table 2. The primer sequences for analysed genes.

Gene	Primer	Sequence (5′–3′)	Tm (°C)	Product Size (nt)	Gen Bank Access No.
ACTB	Forward	CCCGCGAGTACAACCTTCTTG	61.27	71	NM_031144.3
	Reverse	GTCATCCATGGCGAACTGGTG	61.61		
GAPDH	Forward	CCGCATCTTCTTGTGCAGTG	59.83	79	NM_017008.4
	Reverse	CGATACGGCCAAATCCGTTC	59.42		
ZO-1	Forward	GGAGCGGGGACAAGATGAAG	60.46	123	XM_039105296.1
	Reverse	AGGATGGAGTTACCCACAGC	59.09		
OCLN	Forward	GGGGCGCAGCAGGTCT	61.91	181	NM_031329.3
	Reverse	GTGCATCTCTCCGCCATACA	59.90		
TFF2	Forward	ACGCCCTCCAACAGAAAGAA	59.53	140	NM_053844.2
	Reverse	CATTGTTCCGACGCTTGGTT	59.41		
OGG1	Forward	GACATCGCACCCTAACCTCC	60.18	118	NM_030870.1
	Reverse	CTTTGCTCCCTCCACCGGAA	62.12		

ACTB, β-actin; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *ZO-1*, zonula occludens-1; *OCLN*, occludin; *TFF2*, trefoil factor 2; *OGG1*, 8-oxoguanine glycosylase.

2.5. Histological Examination of Organs

Histopathological examinations of small intestine samples from rats were performed according to the procedure described by Cholewińska et al. [31].

2.6. Data Analysis and Statistics

The STATISTICA software, version 12.0 (StatSoft Corp., Krakow, Poland), was used to determine the differences among treatment groups. Two-way ANOVA was applied to assess the effects of main factors CuNPs dose (L, 6.5 mg/kg and H, 13 mg/kg) and dietary fibre type (cellulose, pectin, inulin and psyllium), followed by Duncan's multiple range test. Additionally, each experimental group fed CuNPs L dose was compared with the control C group (fed diet with 6.5 mg/kg Cu from $CuCO_3$ and containing cellulose as the main dietary fibre source) with the aid of a *t*-test. Similarly, the *t*-test was used to compare the experimental groups fed diets with CuNPs H dose with the control CH group fed diet with 13 mg/kg Cu from $CuCO_3$ and containing cellulose as the main dietary fibre source. Differences with $p \leq 0.05$ are considered to be significant.

3. Results

3.1. One-Way Analysis of Variance (ANOVA)

3.1.1. C vs. CN, PN, JN and SN

(The C group was fed a control diet with standard Cu content in the mineral mixture (6.5 mg/kg) from CuCO3 with 8% of cellulose as dietary fibre source; the CN group was fed a diet with a supplementation of 6.5 mg Cu/kg from CuNPs with 8% of cellulose dietary fibre source; the PN group was fed a diet with a supplementation of 6.5 mg Cu/kg from CuNPs with 2% of cellulose and 6% of pectin dietary fibre source; the JN group was fed a diet with a supplementation of 6.5 mg Cu/kg from CuNPs with 2% of cellulose and 6% of inulin dietary fibre source; and the SN group was fed a diet with a supplementation of 6.5 mg Cu/kg from CuNPs with 2% of cellulose and 6% of psyllium dietary fibre source).

At the beginning of the experiment, a significant reduction in daily weight gain was observed in the JN and SN groups. The JN group also showed a significant reduction in daily dietary intake, with a simultaneous increase in kidney weight compared to the control (Table 3). There was also an increase in the weight of the small intestine with its content in the PN, JN and SN groups; the ileal viscosity in the PN and SN groups; as well as the tissue and contents weights of the cecum in the JN and SN groups. Reduced ileal DM was also observed in the PN and JN groups compared to the control. In the PN group, a lower pH of the ileum content was found, while in the SN group, the value of this parameter was increased compared to control (Table 4). An increased DAO activity in serum was also found in the JN group. In the blood plasma of all compared experimental groups (CN, PN,

JN and SN), a reduced content of lactic acid was noted (Table 5). In the tissue of the small intestine of PN group rats, a significant decrease in the level of APE-1 was noted, while in the CN group, the level of lactic acid was significantly reduced compared to the control. In the small intestine tissue of rats from all compared experimental groups (CN, PN, JN and SN), reduced levels of Caspase-3 and Caspase-8 were demonstrated (Table 6). Moreover, a decrease in the level of expression of the *OGG1* gene in the CN, JN and SN experimental groups; the *TFF2* gene in the CN, PN and SN experimental groups; and the *ZO-1* gene in the CN, PN and JN experimental groups compared to control group was found (Table 7).

Table 3. Body weight, feed intake, NMR body composition and internal organs weight in rats fed experimental diets (n = 10 per group) *.

	Initial BW	Final BW	BW Gain	BW Gain	Intake	Heart	Spleen	Kidneys	Body Fat ^	Body Lean ^	Body Fluids ^
	g	g	g	g/day	g/day	g/100 g BW	g/100 g BW	g/100 g BW	%	%	%
Control C	278	396	118	2.91	19.0	0.245	0.183	0.547	13.4	61.6	25.1
Control CH	278	394	116	2.86	18.8	0.243	0.175	0.557	12.7	61.2	24.2
2-way ANOVA:											
CN	278	398	121	2.97	19.1	0.247	0.184	0.542	12.2	62.9	24.9
CNH	278	393	116	2.83	19.1	0.246	0.190 $^{\&}$	0.542	12.9	62.4	24.7
PN	278	395	118	2.86	18.7	0.252	0.181	0.556	12.6	61.9	25.5
PNH	278	398	120	2.92	18.9	0.243	0.181	0.558	13.0	63.0	24.0
JN	278	379	101	2.46 $^{\#}$	17.5 $^{\#}$	0.255	0.202	0.578 $^{\#}$	13.0	62.4	24.6
JNH	278	389	112	2.72	18.3	0.246	0.189 $^{\&}$	0.562	12.8	62.5	24.6
SN	278	385	107	2.58 $^{\#}$	18.4	0.250	0.194	0.573	12.8	63.5	24.1
SNH	278	386	108	2.61	18.2	0.247	0.180	0.564	12.8	62.2	25.0
SEM	1.047	1.725	1.851	0.042	0.110	0.002	0.002	0.004	0.193	0.426	0.503
Cu-NP dose (D)											
L (6.5 mg/kg)	277	389	112	2.72	18.4	0.251	0.190	0.562	12.6	62.7	24.8
H (13 mg/kg)	277	392	114	2.76	18.6	0.246	0.185	0.556	12.9	62.5	24.6
p value	0.888	0.559	0.648	0.626	0.346	0.243	0.352	0.544	0.487	0.895	0.882
Fibre type (F)											
C (cellulose)	277	395	118	2.88 ^{a}	19.1 ^{a}	0.249	0.187	0.542	12.5	62.5	24.8
P (pectin)	278	397	119	2.89 ^{a}	18.8 ^{ab}	0.247	0.181	0.557	12.8	62.4	24.8
J (inulin)	278	384	107	2.59 ^{b}	17.9 ^{c}	0.251	0.196	0.570	12.9	62.5	24.6
S (psyllium)	279	386	108	2.59 ^{b}	18.3 ^{bc}	0.248	0.187	0.569	12.6	62.8	24.5
p value	0.998	0.051	0.078	0.028	0.004	0.951	0.322	0.110	0.925	0.991	0.998
Interaction D × F											
p value	0.999	0.561	0.575	0.475	0.479	0.778	0.534	0.884	0.917	0.861	0.913

* The dietary treatments used in the experimental feeding period: groups C and CH were fed a control diet with standard and enhanced Cu content in the mineral mixture (6.5 and 13 mg/kg from $CuCO_3$, respectively) with 8% of cellulose as a dietary fibre source; groups CN and CNH were fed diets with a supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 8% of cellulose as a dietary fibre source; groups PN and PNH were fed diets with a supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of pectin as a dietary fibre source; groups JN and JNH were fed diets with a supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of inulin as a dietary fibre source; groups SN and SNH were fed diets with a supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of psyllium as a dietary fibre source; L was given treatment (n = 40) with dietary CuNPs 6.5 mg/kg dose; H was given treatment (n = 40) with dietary CuNPs 13 mg/kg dose; C was given treatment (n = 20) with cellulose as dietary fibre; P was given treatment (n = 20) with pectin as dietary fibre; J was given treatment (n = 20) with inulin as dietary fibre; S was given treatment (n = 20) with psyllium as dietary fibre; ^{a,b,c} Mean values within a column with unlike superscript letters are shown to be significantly different ($p < 0.05$); differences between the 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 6.5 mg/kg (CN, PN, JN, SN) was compared with the control C one with the aid of *t*-test ($^{\#}$ indicates a significant difference versus the C group); similarly, each experimental group fed CuNPs 13 mg/kg (CNH, PNH, JNH, SNH) was compared with the control CH with the aid of a *t*-test ($^{\&}$ indicates a significant difference versus the CH group); BW, body weight; SEM, pooled standard error of mean (standard deviation for all rats divided by the square root of rat number, n = 100). ^ Nuclear magnetic resonance (NMR) analysis.

Table 4. Intestinal parameters in rats fed experimental diets (n = 10 per group) *.

	Small Intestine with Contents	Ileal Viscosity	Ileal DM	Ileal pH
	g/100 g BW	mPa·s	%	
Control C	1.37	1.50	19.3	7.14
Control CH	1.38	1.55	19.4	7.15
2-way ANOVA:				
CN	1.33	1.54	19.3	7.22
CNH	1.34	1.51	19.7	7.23
PN	1.49 $^{\#}$	2.72 $^{\#}$	14.3 $^{\#}$	7.01 $^{\#}$
PNH	1.52 $^{\&}$	2.76 $^{\&}$	15.0 $^{\&}$	7.06
JN	1.49 $^{\#}$	1.59	18.1 $^{\#}$	7.15
JNH	1.57 $^{\&}$	1.66	18.0 $^{\&}$	7.09
SN	1.82 $^{\#}$	2.88 $^{\#}$	18.5	7.25 $^{\#}$
SNH	1.78 $^{\&}$	2.95 $^{\&}$	17.6 $^{\&}$	7.23 $^{\&}$
SEM	0.019	0.068	0.217	0.012
Cu-NP dose (D)				
L (6.5 mg/kg)	1.53	2.18	17.5	7.15
H (13 mg/kg)	1.55	2.22	17.6	7.15
p value	0.436	0.581	0.937	0.891
Fibre type (F)				
C (cellulose)	1.34 ^{c}	1.53 ^{b}	19.5 ^{a}	7.22 ^{a}
P (pectin)	1.50 ^{b}	2.74 ^{a}	14.6 ^{c}	7.03 ^{c}
J (inulin)	1.53 ^{b}	1.63 ^{b}	18.0 ^{b}	7.12 ^{b}
S (psyllium)	1.80 ^{a}	2.91 ^{a}	18.0 ^{b}	7.24 ^{a}
p value	<0.001	<0.001	<0.001	<0.001
Interaction D × F				
p value	0.323	0.924	0.234	0.463

* The dietary treatments used in the experimental feeding period: groups C and CH were fed a control diet with standard and enhanced Cu content in the mineral mixture (6.5 and 13 mg/kg from $CuCO_3$, respectively) with 8% of cellulose as dietary fibre source; groups CN and CNH were fed diets with a supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 8% of cellulose dietary fibre source; groups PN and PNH were fed diets with a supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of pectin as a dietary fibre source; groups JN and JNH were fed diets with a supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of inulin as a dietary fibre source; groups SN and SNH were fed diets with a supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of psyllium as a dietary fibre source; L was given treatment (n = 40) with dietary Cu-NP 6.5 mg/kg dose; H, treatment (n = 40) with dietary CuNPs 13 mg/kg dose; C was given treatment (n = 20) with cellulose as dietary fibre; P was given treatment (n = 20) with pectin as dietary fibre; J was given treatment (n = 20) with inulin as dietary fibre; S was given treatment (n = 20) with psyllium as dietary fibre; ^{a,b,c} Mean values within a column with unlike superscript letters are shown to be significantly different ($p < 0.05$); differences between the 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 6.5 mg/kg (CN, PN, JN, SN) was compared with the control C with the aid of a *t*-test ($^{\#}$ indicates a significant difference versus the C group); similarly, each experimental group fed CuNPs 13 mg/kg (CNH, PNH, JNH, SNH) was compared with the control CH with the aid of a *t*-test ($^{\&}$ indicates a significant difference versus the CH group); BW, body weight; SEM, pooled standard error of mean (standard deviation for all rats divided by the square root of rat number, n = 100). Ileal DM, ileal dry matter.

Table 5. Blood plasma parameters in rats fed experimental diets (n = 10 per group) *.

	DAO	Lactic Acid
	mIU/mL	ng/mL
Control C	8.63	26.2
Control CH	10.5	22.9
2-way ANOVA:		
CN	8.03	17.3 $^{b\#}$
CNH	9.86	21.6 ^{a}
PN	9.20	21.8 $^{a\#}$
PNH	10.2	20.1 $^{a\&}$

Table 5. *Cont.*

	DAO	Lactic Acid
	mIU/mL	ng/mL
JN	9.85 #	21.2 a#
JNH	10.7	20.2 a&
SN	9.67	21.4 a#
SNH	10.9	20.8 a
SEM	0.177	0.314
Cu-NP dose (D)		
L (6.5 mg/kg)	9.19 b	20.5
H (13 mg/kg)	10.4 a	20.7
p value	0.001	0.669
Fibre type (F)		
C (cellulose)	8.94 b	19.5
P (pectin)	9.70 ab	21.0
J (inulin)	10.3 a	20.7
S (psyllium)	10.3 a	21.1
p value	0.036	0.128
Interaction D × F		
p value	0.808	<0.001

* The dietary treatments used in the experimental feeding period: groups C and CH were fed a control diet with standard and enhanced Cu content in the mineral mixture (6.5 and 13 mg/kg from $CuCO_3$, respectively) with 8% of cellulose as a dietary fibre source; groups CN and CNH were fed diets with a supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 8% of cellulose as a dietary fibre source; groups PN and PNH were fed diets with a supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of pectin as a dietary fibre source; groups JN and JNH were fed diets with a supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of inulin as a dietary fibre source; groups SN and SNH were fed diets with a supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of psyllium as a dietary fibre source; L was given treatment (n = 40) with dietary CuNPs 6.5 mg/kg dose; H was given treatment (n = 40) with dietary CuNPs 13 mg/kg dose; C was given treatment (n = 20) with cellulose as dietary fibre; P was given treatment (n = 20) with pectin as dietary fibre; J was given treatment (n = 20) with inulin as dietary fibre; S was given treatment (n = 20) with psyllium as dietary fibre; [a,b] Mean values within a column with unlike superscript letters are shown to be significantly different ($p < 0.05$); differences between the 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 6.5 mg/kg (CN, PN, JN, SN) was compared with the control C with the aid of a *t*-test ([#] indicates a significant difference versus the C group); similarly, each experimental group fed CuNPs 13 mg/kg (CNH, PNH, JNH, SNH) was compared with the control CH with the aid of a *t*-test ([&] indicates a significant difference versus the CH group); SEM, pooled standard error of mean (standard deviation for all rats divided by the square root of rat number, n = 100). DAO, diamine oxidase.

Table 6. Small intestinal biochemical parameters in rats fed experimental diets (n = 10 per group) *.

	APE-1	OGG-1	DAO	8-OHdG	Caspase-3	Caspase-8	Lactic Acid
	ng/g	ng/g	mIU/g	ng/g	ng/g	ng/g	ng/g
Control C	202	85.4	128	21.2	20.9	47.1	5.17
Control CH	195	74.0	121	25.5	16.1	36.2	4.48
2-way ANOVA:							
CN	219 a	89.2	138	28.2#	17.0 #	35.1 #	3.56 c#
CNH	205 ab	78.9	131	25.6	16.3	30.4 &	3.81 c
PN	104 d#	77.5	130	22.4	12.3 #	35.7 #	6.24 a
PNH	188 bc	92.1 &	132	23.8	15.6	32.6	4.39 c
JN	142 cd	94.4	133	28.2 #	17.5 #	33.6 #	5.48 ab
JNH	161 bc&	99.7 &	124	27.5	16.0	33.9	5.78 a&
SN	169 bc	96.3	158	25.3	14.5 #	33.5 #	5.32 ab
SNH	196 ab	94.2 &	118	26.9	15.1	39.2	4.13 c
SEM	5.792	1.805	3.779	0.672	0.417	0.942	0.151
Cu-NP dose (D)							
L (6.5 mg/kg)	159	89.4	140	26.0	15.3	34.5	5.15
H (13 mg/kg)	188	91.2	127	25.9	15.8	34.0	4.53

Table 6. *Cont.*

	APE-1	OGG-1	DAO	8-OHdG	Caspase-3	Caspase-8	Lactic Acid
	ng/g	ng/g	mIU/g	ng/g	ng/g	ng/g	ng/g
p value	0.010	0.634	0.151	0.948	0.584	0.820	0.032
Fibre type (F)							
C (cellulose)	212	84.1 ^{b}	135	26.9	16.6 ^{a}	32.7	3.69
P (pectin)	146	84.8 ^{b}	131	23.1	14.0 ^{b}	34.2	5.31
J (inulin)	152	97.1 ^{a}	129	27.9	16.8 ^{a}	33.8	5.63
S (psyllium)	182	95.3 ^{ab}	138	26.1	14.8 ^{ab}	36.3	4.72
p value	<0.001	0.035	0.898	0.062	0.043	0.642	<0.001
Interaction D × F							
p value	0.020	0.151	0.409	0.606	0.186	0.294	0.019

* The dietary treatments used in the experimental feeding period: groups C and CH were fed a control diet with standard and enhanced Cu content in the mineral mixture (6.5 and 13 mg/kg from $CuCO_3$, respectively) with 8% of cellulose as a dietary fibre source; groups CN and CNH were fed diets with supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 8% of cellulose as a dietary fibre source; groups PN and PNH were fed diets with supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of pectin as a dietary fibre source; groups JN and JNH were fed diets with supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of inulin as a dietary fibre source; groups SN and SNH were fed diets with supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of psyllium as a dietary fibre source; L was given treatment (n = 40) with dietary CuNPs 6.5 mg/kg dose; H, treatment (n = 40) with dietary CuNPs 13 mg/kg dose; C was given treatment (n = 20) with cellulose as dietary fibre; P was given treatment (n = 20) with pectin as dietary fibre; J was given treatment (n = 20) with inulin as dietary fibre; S was given treatment (n = 20) with psyllium as dietary fibre; ^{a,b,c,d} Mean values within a column with unlike superscript letters are shown to be significantly different ($p < 0.05$); differences between the 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 6.5 mg/kg (CN, PN, JN, SN) was compared with the control C with the aid of a *t*-test ($^{\#}$ indicates a significant difference versus the C group); similarly, each experimental group fed CuNPs 13 mg/kg (CNH, PNH, JNH, SNH) was compared with the control CH with the aid of a *t*-test ($^{\&}$ indicates a significant difference versus the CH group); SEM, pooled standard error of mean (standard deviation for all rats divided by the square root of rat number, n = 100). APE-1, apurinic/apyrimidinic endonuclease 1; OGG1, 8-oxoguanine glycosylase; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; DAO, diamine oxidase.

Table 7. Level of gene expression in the small intestine of rats fed experimental diets (n = 10 per group) *.

	OCLN	*OGG1*	*TFF2*	*ZO-1*
Control C	1.34	1.92	1.15	1.05
Control CH	0.543	0.279	0.306	0.215
2-way ANOVA:				
CN	0.729	0.087 $^{b\#}$	0.326 $^{\#}$	0.407 $^{\#}$
CNH	0.947	0.279 ^{ab}	0.306	0.311
PN	0.651	0.719 ^{a}	0.458 $^{\#}$	0.468 $^{\#}$
PNH	0.902	0.110 ^{b}	0.402 $^{\&}$	0.518 $^{\&}$
JN	0.966	0.346 $^{ab\#}$	0.750	0.337 $^{\#}$
JNH	0.672	0.379 ^{ab}	0.361	0.605 $^{\&}$
SN	0.958	0.139 $^{b\#}$	0.305 $^{\#}$	0.761
SNH	1.13 $^{\&}$	0.240 $^{b\&}$	0.511 $^{\&}$	0.646 $^{\&}$
SEM	0.073	0.093	0.052	0.039
Cu-NP dose (D)				
L (6.5 mg/kg)	0.826	0.323	0.460	0.493
H (13 mg/kg)	0.912	0.252	0.395	0.520
p value	0.552	0.501	0.501	0.713
Fibre type (F)				
C (cellulose)	0.838	0.183	0.316	0.359 ^{b}
P (pectin)	0.776	0.414	0.430	0.493 ^{b}
J (inulin)	0.819	0.362	0.556	0.471 ^{b}
S (psyllium)	1.04	0.190	0.408	0.704 ^{a}
p value	0.567	0.289	0.370	0.013

Table 7. *Cont.*

	OCLN	*OGG1*	*TFF2*	*ZO-1*
Interaction D × F *p* value	0.504	0.037	0.189	0.228

* The dietary treatments used in the experimental feeding period: groups C and CH were fed a control diet with standard and enhanced Cu content in the mineral mixture (6.5 and 13 mg/kg from $CuCO_3$, respectively) with 8% of cellulose as dietary fibre source; groups CN and CNH were fed diets with a supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 8% of cellulose as a dietary fibre source; groups PN and PNH were fed diets with a supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of pectin as a dietary fibre source; groups JN and JNH were fed diets with a supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of inulin as a dietary fibre source; groups SN and SNH were fed diets with a supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of psyllium as a dietary fibre source; L was given treatment (n = 40) with dietary CuNPs 6.5 mg/kg dose; H was given treatment (n = 40) with dietary CuNPs 13 mg/kg dose; C was given treatment (n = 20) with cellulose as dietary fibre; P was given treatment (n = 20) with pectin as dietary fibre; J was given treatment (n = 20) with inulin as dietary fibre; S was given treatment (n = 20) with psyllium as dietary fibre; [a,b] Mean values within a column with unlike superscript letters are shown to be significantly different ($p < 0.05$); differences between the 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 6.5 mg/kg (CN, PN, JN, SN) was compared with the control C with the aid of a *t*-test ([#] indicates a significant difference versus the C group); similarly, each experimental group fed CuNPs 13 mg/kg (CNH, PNH, JNH, SNH) was compared with the control CH with the aid of a *t*-test ([&] indicates a significant difference versus the CH group); SEM, pooled standard error of mean (standard deviation for all rats divided by the square root of rat number, n = 100). *OCLN*, occluding; *OGG1*, 8-oxoguanine glycosylase; *TFF2*, trefoil factor 2; *ZO-1*, zonula occludens-1.

3.1.2. CH vs. CNH, PNH, JNH and SNH

(The CH group was fed a control diet with enhanced Cu content in the mineral mixture (13 mg/kg from $CuCO_3$) with 8% of cellulose as a dietary fibre source; the CNH group was fed a diet with a supplementation of 13 mg Cu/kg from CuNPs, with 8% of cellulose as a dietary fibre source; the PNH group was fed a diet with a supplementation of 13 mg Cu/kg from CuNPs with 2% of cellulose and 6% of pectin as a dietary fibre source; the JNH group was fed a diet with a supplementation of 13 mg Cu/kg from CuNPs with 2% of cellulose and 6% of inulin as a dietary fibre source; the SNH group was fed a diet with a supplementation of 13 mg Cu/kg from CuNPs with 2% of cellulose and 6% of psyllium as a dietary fibre source).

Next, a significant increase in spleen weight was observed in the CNH and JNH groups (Table 3). In addition, a significant increase in the weight of the small intestine content and a decrease in the ileal DM in the PNH, JNH and SNH groups was noted. Compared to the CH group, the ileal viscosity in the PNH and SNH groups was increased. Moreover, an increase in the pH of the ileum content was noted in the SNH group (Table 4). Decreased lactic acid level in the serum was noted in the PNH and JNH groups vs. CH (Table 5). In all experimental groups (PNH, JNH and SNH), an increase in the level of OGG-1 in the tissue of the small intestine was observed. Moreover, in the examined tissue of rats from the JNH group, a decrease in the level of APE-1 was also noted, with a simultaneous decrease in the content of lactic acid compared to the CH control group. A reduction in the content of Caspase-8 was also found in the small intestine of rats from the CNH group vs. the CH group (Table 6). In the small intestine of SNH rats, an increase in the expression of *OCLN* and *TFF2* genes was observed, while a decrease in the expression of the *OGG1* gene was noted compared to CH control group. Intestinal *TFF2* gene level in the PNH group and *ZO-1* gene expression level in the PNH, JNH and SNH groups were higher compared to CH control (Table 7).

3.2. Two-Way Analysis of Variance (ANOVA)

The interactions for plasma lactic acid content were noted ($p < 0.001$; Table 5), as well as APE-1 and lactic acid levels ($p = 0.020$ and $p = 0.019$, respectively; Table 6) and *OGG1* gene expression ($p = 0.037$; Table 7). The occurrence of these interactions indicates that the main effects did not have a significant effect on the parameters that were studied. The

interaction observed for the level of lactic acid ($p < 0.001$) in the blood plasma of rats is related to the fact that the use of a higher dose of CuNPs resulted in an increase in the content of this indicator in rats from the CN group, which was not observed when using a higher dose of CuNPs in the PN, JN and SN groups (Table 5). The interaction observed for the level of APE-1 ($p = 0.020$) in the tissue of the small intestine of rats is due to the fact that the use of a higher dose of CuNPs resulted in an increase in the level of this enzyme in rats from the PN, JN and SN groups, which was not observed with the use of higher dose CuNPs in the CN group. In turn, the interaction observed for the content of lactic acid ($p = 0.019$) in the small intestine of rats is related to the fact that the use of a higher dose of CuNPs resulted in a decrease in the content of this indicator in the PN and SN groups, which was not observed when using a higher dose of CuNPs in the CN and JN groups (Table 6). The interaction observed for the intestinal *OGG1* expression level ($p = 0.037$) is related to the fact that the use of a higher dose of CuNPs resulted in a decrease in these parameters in rats from the PN group, which was not observed when using a higher dose of CuNPs in the CN, JN and SN groups (Table 7).

3.2.1. Effect of CuNPs Dose

The introduction of twice as high levels of CuNPs into the diet did not have a significant effect on the increased level of Cu in the blood plasma, because the values recorded in individual groups were in the range of 89.9–111 µmol/l (unpublished data). Increasing the level of CuNPs from 6.5 to 13 mg/kg diet in the rat diet resulted in an increase DAO levels in the blood ($p = 0.001$; Table 5).

3.2.2. Effect of Dietary Fibre Type

Further, it was observed that feeding rats with a diet containing inulin or psyllium resulted in a decrease in daily weight gain and daily dietary intake ($p = 0.028$ and $p = 0.004$, respectively) compared to the control receiving cellulose as a standard dietary fibre source (Table 3). Importantly, the results were independent of the level of CuNPs used. Including pectin, inulin or psyllium fibres resulted in an increase in the weight of the small intestine and its contents, while reducing the ileal DM ($p < 0.001$, both) compared to cellulose only. The addition of pectin or inulin lowered the pH of the ileal contents of rats ($p < 0.001$) also. Supplementation of pectin or psyllium also resulted in an increase in ileal viscosity ($p < 0.001$; Table 4). In addition, reduced levels of DAO were found in the blood serum of rats fed a diet supplemented with inulin or psyllium ($p = 0.036$; Table 5). Including inulin resulted in an increase in the level of OGG-1 ($p = 0.035$), while the inclusion of pectin to the diet contributed to a decrease in the level of caspase-3 ($p = 0.043$) in the small intestine compared to being control fed a diet containing cellulose only (Table 6). In the small intestine of rats receiving a diet enriched with psyllium, a higher level of *ZO-1* ($p = 0.013$) gene expression than in the control rats was also observed (Table 7).

3.3. Histology Examination of Small Intestine

The histopathological examination showed a physiological structure of the small intestine with the presence of small–insignificant tissue defects in the apical parts of the villi in rats from C and CH groups (Figure 1A,B, respectively). The normal, physiological structure of the small intestine was found for the CN and PN groups (Figure 1C,E, respectively). In the small intestine of rats from the JN and SN groups, single–significant tissue defects in the apical parts of the villi were found (Figure 1G,I, respectively). In the small intestine of rats from the CNH, PNH, JNH and SNH groups, damage to the apical part of the villi was numerous and significant, wherein these lesions were the most severe in the CNH group (Figure 1D,F,H,J, respectively). No pathological changes at the base of the villi and in the mucosa of the small intestine were found in any of the examined groups of rats.

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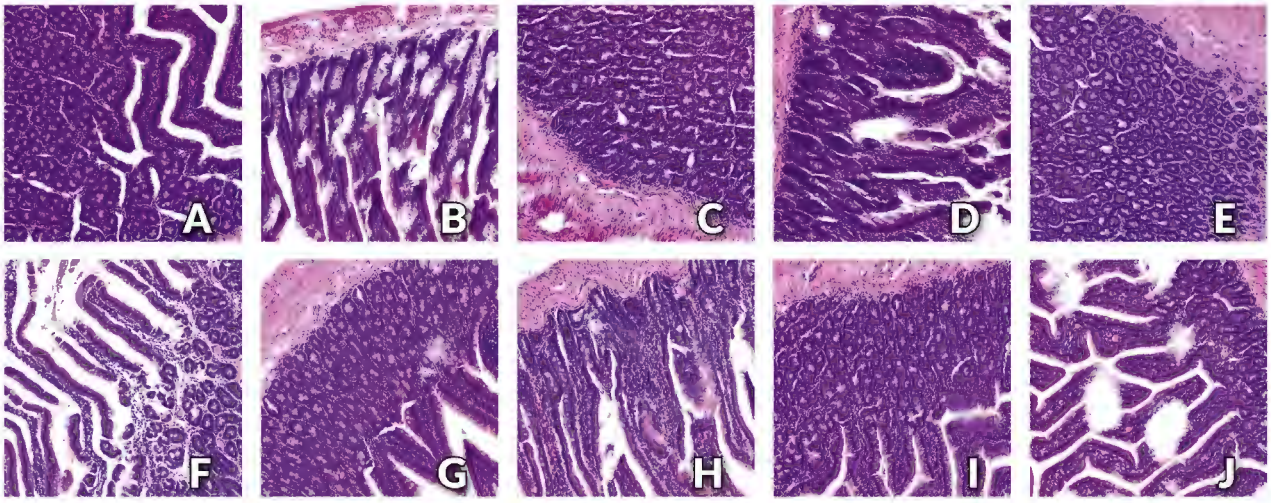

Figure 1. Morphological effects of different Cu sources ($CuCO_3$ or CuNPs), CuNPs levels (L—6.5 mg Cu/kg diet or H—13.0 mg Cu/kg diet) or type of fibre (C—cellulose, P—pectin, J—inulin or S—psyllium) in the diet on the small intestine of rats; 10× magnification. Treatments: (**A**)—C, (**B**)—CH, (**C**)—CN, (**D**)—CNH, (**E**)—PN, (**F**)—PNH, (**G**)—JN, (**H**)—JNH, (**I**)—SN, (**J**)—SNH.

4. Discussion

The results of the study did not show that the replacement of the recommended level of Cu by CuNPs affected the diet intake and body weight of experimental rats, which is fully consistent with the results of our previous studies on rats [11]. There were also no significant changes in the analysed growth parameters in response to the increasing CuNPs level. However, a decrease consumption and body weight were noted in rats whose diet was supplemented with inulin or psyllium. Others also report such observations [25,34–39]. An explanation may be that dietary fibre naturally increases the feeling of satiety, consequently reducing the overall caloric intake [37]. The observed effects caused by inulin or psyllium may be related to the fact that both forms dissolve well in water, forming a gel in the small intestine, which prolongs the feeling of satiety, thus reducing the intake of energy and weight loss, or to the specific rheological properties of high-fibre foods that require longer chewing [40]. Adam et al. [37] prove that soluble dietary fibre of a prebiotic nature stimulates the secretion of satiety hormones, such as glucagon-like peptide-1 (GLP-1) and tyrosine-tyrosine peptide (PYY), by the mucosa of the small intestine, which effectively suppresses appetite and reduces the food intake. Moreover, in their studies, the authors have also shown that the stimulation of the secretion of satiety hormones may occur as a result of the activation of receptors located in the small intestine by an increased amount of signalling short-chain fatty acids (SCFAs) produced during fibre fermentation [37]. Interestingly, despite the significant role of pectin in dietary intake and body weight reduction [37], the results of our study did not confirm the significant effect of this form of fibre on the reduction of growth parameters in rats. In turn, our results are consistent with others [41,42], and proved that a diet containing pectin is well tolerated, and no changes in the amount of food consumed or body weight were noted. Taken together, the results presented here and elsewhere suggest that the observed effects are correlated with dietary pectin levels. It seems possible that the inclusion of a higher proportion of this type of fibre in the diet could result in a decrease in the growth rate of rats tested.

Further, the replacement of $CuCO_3$ by CuNPs, as well as a two-fold increase in their level compared to dietary recommendations had no effect on intestinal parameters, such as small intestine weight with contents, and the dry matter, viscosity or pH of the ileum. However, these parameters can be modulated by introducing alternative forms of dietary fibre to the diet. The results of our study showed an increase in the weight of the small intestine with its content in rats fed a diet containing pectin, inulin or psyllium. Our results

are consistent with others [42–45]. Dongowski et al. [42] noted an increase in the weight of the ileum tissue and its content in rats fed a diet containing pectin. A similar increase in the weight of the small intestine in rats fed a diet containing pectin for 20 days was also noted by Pirman et al. [46]. Moreover, Krupa-Kozak et al. [45] proved that a gluten-free diet supplemented with prebiotic inulin for 6 weeks also showed a significant increase the relative weight of the small intestine in rats. Arjmandi et al. [43] observed an increase in the relative weight of the small intestine in rats fed a diet containing cholesterol and 5 or 10% psyllium for 21 days, which was stored at 5 °C or 40 °C for 8 months, compared to a control group receiving a diet containing 10% cellulose. Kristensen et al. [44] also observed analogous changes as a significant increase in the weight of the gastrointestinal tract in total and its individual segments, including the small intestine, in rats receiving a dietary fibre supplement from linseed. Dongowski et al. [42] explain the obtained effect by the fact that dietary fibre (especially pectin) is not or only slightly degraded in the small intestine of rats, which means that the fibre contained in the gastrointestinal content binds water, thereby increasing its viscosity. The small intestine has a difficult task because it has to transport this sticky content to the lower segments of the digestive tract, which causes its cells to grow, and, as a consequence, its mass also increases. Although our study confirmed an increase in ileal viscosity in rats fed a diet supplemented with pectin and psyllium, histological evaluation did not reveal an overgrowth of the small intestinal wall cells in any of the experimental groups. In our study, an increase in the acidity of the ileum of rats fed a diet with the supplementation of pectin and inulin was also shown. Krupa-Kozak et al. [45] suggest that an increase in the weight of the small intestine with simultaneous acidification of its environment may indicate that inulin (and perhaps other soluble forms of fibre) may be metabolised to some extent by air-tolerant small intestine bacteria. The increased weight of the small intestine with its content obtained in our research, with a simultaneous reduction in the dry matter of the ileum in rats from all experimental groups, and an increase in the viscosity of the small intestine of rats receiving pectin or psyllium, allows us to assume that the observed changes may be primarily the result of hydration properties of dietary fibre. All three forms of tested dietary fibre (inulin, pectin and psyllium) have a tendency to dissolve in water and bind it, wherein pectin forms very viscous solutions, and psyllium swells when binding water [37]. Food containing fibre, passing through subsequent sections of the digestive tract, up to the small intestine, binds water while increasing its volume and weight. Taking into account the significant increase in the total weight of the small intestine with the simultaneous decrease in the dry matter of the ileum in all experimental groups, it may be assumed that these differences were affected by the weight of water bound by dietary fibre present in the intestinal content.

In the assessment of the intestinal barrier function, diamine oxidase (DAO) is often considered. This enzyme catalyses the decomposition reaction of histamine in the digestive tract, which is responsible for the development of an allergic reaction. Under physiological conditions, a relatively high level of DAO is found in the small intestine, while in blood plasma it is very low. When the intestinal mucosa is damaged, e.g., as a result of ischemia, hypoxia, contact with a harmful factor present in food or tissue nutrition disorders, an inflammatory reaction is triggered with the release of histamine. Due to the necessity of its deactivation, a decrease in the level of DAO in the intestinal mucosa is observed, resulting in a simultaneous decrease in the level of this indicator in the blood [47]. Our results indicate that the replacement of Cu in the standard form of $CuCO_3$ by an equivalent dose of CuNPs had no effect on the DAO level in the blood plasma and small intestine. However, the level of DAO in the blood plasma increased with no changes in the level of this indicator in the small intestine as a result of increasing the CuNPs dietary level. Surprisingly, the results of our study also showed no significant differences in the level of intestinal DAO between the experimental groups and control group fed a diet containing the addition of cellulose, while the value of this indicator was significantly increased in the blood plasma of rats receiving a diet containing the addition of inulin or psyllium. Although the obtained results are ambiguous, they undoubtedly indicate a beneficial effect of both the introduction of a

doubled dose of CuNPs compared to the standard recommendation, as well as alternative forms of fibre (inulin and psyllium) on maintaining a proper intestinal barrier.

Lactic acid is the end product of glucose oxidation in anaerobic glycolysis [48]. It may be synthesised in situ on the intestinal mucosa by anaerobic bacteria colonising the lumen of the large intestine, such as lactobacilli, streptococci and bifidobacteria [49,50]. There are reports that this compound may affect a number of metabolic and immunological processes in the body, including mediation in the signalling pathways; the production of pro- and anti-inflammatory mediators by T lymphocytes and macrophages; or by affecting the redox status through the reaction of lactate dehydrogenase inducing reactive oxygen species and acting as an inhibitor of glucose breakdown [49]. Okada et al. [51], in studies on mice, also proved that lactate can stimulate the proliferation of enterocytes, which has a positive effect on maintaining the function of the intestinal barrier. However, there are also studies that found an increase in the synthesis of lactic acid in the intestines and the accompanying discharge of this compound into the blood, which may be observed in the case of intestinal hypoxia, resulting in the intensification of anaerobic glucose breakdown. As a consequence, it may lead to dangerous lactic acidosis development [48]. The results of our study indicate that replacing the traditional $CuCO_3$ with CuNPs resulted in a reduction in the level of lactic acid in the small intestine and blood serum as well. In light of the above-mentioned reports, this effect may be considered positive. In addition, the increased lactic acid level in the intestine may result in acidification of the environment. In the case of the large intestine, this effect may be desirable, because it protects it, among others, from colonisation by pathogenic bacteria [52], while, in the case of the small intestine, this effect may be completely different. Digestive enzymes secreted into the lumen of the small intestine require a slightly alkaline environment for their activation and proper functioning [53]. Acidification of the environment caused by the overproduction of lactic acid in the small intestine may therefore result in a reduction in digestive processes, and thus, in the efficiency of the absorption of nutrients. This also seems to confirm the beneficial effect of CuNPs on the functioning of the small intestine. Moreover, our study also noted a significant DxF interaction for LA. This results from the fact that, in the case of the combined use of pectin or psyllium as a source of fibre with a higher level of CuNPs, a reduced level of this indicator was found in the intestinal wall, which was not observed in the case of the combined use of nanoparticles with cellulose or pectin. It is likely that the use of a higher level of CuNPs with the above-mentioned sources of fibre has a more beneficial effect on intestinal integrity than its combination with cellulose, as also indicated by the increased level of LA in the blood plasma of rats from the cellulose group, together with a higher level of CuNPs in the diet. Increased production of lactic acid in the small intestine is an unfavourable phenomenon, as it can lead to acidosis, which results in serious damage to the intestinal epithelium, resulting in excessive intestinal permeability, referred to as "leaky gut" [54].

Among all organs, the tissues of the gastrointestinal tract, especially the small intestine, is most exposed to the potentially harmful effects of xenobiotics entering the body through diet. It is assumed that this harmful effect is very often associated with the increased synthesis of free oxygen radicals. These, in turn, contribute to the occurrence of oxidative stress, resulting in damage to cellular macromolecules, including DNA modifications such as the oxidation of nitrogenous bases [55]. In order to prevent the loss of genome integrity, the body has developed various repair systems, among which a very important role is played the DNA repair pathway by cutting out damaged bases (BER) and replacing them with correct ones. This process is initiated by DNA glycosylases, which include, among others, 8-oxoguanine glycosylase (OGG1). This enzyme removes the most common DNA damages such as 8-hydroxydeoxyguanosine (8-OHdG) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) [56]. The results of our previous studies showed no deterioration of the oxidoreductive status of the small intestine in rats due to the replacement of the standard form of Cu by CuNPs [31]. The highly reactive CuNPs may undergo Fenton and/or Haber–Weiss reactions in the body, resulting in the enhanced

production of free radicals, which then damage the genetic material [31]. The results of the present study partially confirm that the replacement of $CuCO_3$ by CuNPs resulted in an increase in the level of 8-OHdG in the small intestine, which suggests the intensification of oxidative processes in the examined tissue. Interestingly, the inclusion of the recommended level of CuNPs in the diet of rats contributed to a downregulation of *OGG1* gene expression in the small intestine, which, however, did not translate into a decrease in the amount of functional OGG1 protein. It was also not found that increasing the level of CuNPs in the diet of rats from 6.5 to 13 mg Cu/kg of diet resulted in a deterioration of the oxidoreductive status and weakening of DNA repair mechanisms in the small intestine. However, an increase in the OGG1 protein level was noted, with no changes in *OGG1* gene expression and 8-OHdG content in rats supplemented with inulin. This suggests that excessive oxidation of nitrogenous bases does not occur, and thus, the OGG1 enzyme is not significantly used for their repair. This allows us to assume that among all the tested forms of fibre, inulin best protects DNA against the harmful effects of free radicals, which may potentially be formed as a result of including CuNPs in the diet. The beneficial effect of the combined use of pectin with a higher level of CuNPs is evidenced by the observed DxF interaction for the level of APE-1 and for the expression of the *OGG1* gene in the small intestine, resulting from the fact that only in the case of combining pectin as a source of fibre with the addition of a higher level of CuNPs, an increase in the level of APE-1 and downregulation of *OGG1* were noted. Although an increase in the APE-1 level (apurinic/apyrimidinic endonuclease 1, one of the DNA repair enzymes) may indicate an increased oxidation of this acid [57]. However, due to the fact that an increased level of 8-OHdG was not observed when a higher level of CuNPs was used together with pectin compared to the use of a lower level of CuNPs with pectin, it should be concluded that the increase in the level of APE-1 was not associated with the induction of the repair mechanism.

In the event of damage to important cellular macromolecules, the programmed cell death may be activated. Caspases play an important role in this multi-stage process [58]. The results of our study indicate that the replacement of $CuCO_3$ by CuNPs resulted in a decrease in caspase 3 and caspase 8 levels in the small intestine of rats, which seems to be a very beneficial phenomenon, and proves that this supplement does not induce significant negative changes in the cells of the small intestine. It also allows us to assume that the previously mentioned induction of oxidative DNA damage as a result of including CuNPs in the diet of rats was not serious enough to lead to significant changes in the genome of intestinal cells, which would result in their being directed to the apoptotic pathway. Moreover, it was not found that increasing the level of CuNPs in the diet contributed to the occurrence of negative changes in the level of the tested caspases. Regardless of the level of CuNPs introduced into the rats' diet, no increase in the synthesis of caspases was found as a result of inulin and psyllium inclusion in the diet, and the addition of pectin reduced the level of caspase 3 in the wall of the small intestine. This suggests that, among the studied forms of fibre, pectin best protects the cells of the small intestine against damage that could lead to their apoptosis.

The intestinal barrier is crucial for maintaining intestinal homeostasis, which determines the proper supply of the body with nutrients and the prevention of intestinal diseases. It consists of the apical cell membrane and intercellular tight junctions (TJ) of enterocytes. Tight junctions are protein complexes resulting from the interaction between members of the claudin family, zonula occludens and MARVEL tight junction proteins (TAMP) [59]. ZO-1 is a cytoplasmic plaque protein that recruits various signalling molecules and acts as a scaffold for transmembrane TJ proteins [60]. This protein is encoded by the *ZO-1* gene, which is expressed especially in the intestine, kidneys, liver, lungs and brain [61]. There are reports that a decrease in ZO-1 protein expression may increase intestinal permeability, which, in turn, may result in the development of intestinal inflammation and even the development of neoplastic lesions [59,62]. It is assumed that Trefoil family factor 2 (TFF2), encoded by the gene of the same name, also plays an important role in maintaining a proper

intestinal barrier [63]. The results of our study showed that *TFF2* may be expressed in the wall of the small intestine. Interestingly, the *TFF2* protein is involved in intestinal defence and repair mechanisms, while *TFF2* overexpression is often observed in the case of significant tissue damage, infection or neoplastic changes in the within the digestive tract [63]. The results of our studies indicate that the replacement of $CuCO_3$ by CuNPs in the diet of rats resulted in *TFF2* and *ZO-1* downregulation, with no changes in the expression level of *OCLN*. However, there was no effect of doubling the level of CuNPs in the diet—in relation to the nutritional recommendations—on the level of expression of the analysed genes of the intestinal barrier in rats. The obtained results can be interpreted in two ways, because the downregulation of *TFF2* gene expression by CuNPs seems to be beneficial, but a decrease in the level of *ZO-1* may indicate that CuNPs increase intestinal permeability and, thus, deteriorate the intestinal barrier. The explanation of the obtained results may be related to the formation of a functional protein, which reduces the expression of the *TFF2* and *ZO-1* mRNA pools. The results of our study also showed that the inclusion of dietary fibre in the form of psyllium in the diet of rats increased the *ZO-1* gene expression in the small intestine, which, in light of reports by other authors, is an effect conducive to maintaining the proper integrity of the intestinal barrier [59,62].

Finally, the results of histopathological examination indicate the normal structure of the small intestine with the presence of small tissue defects in the apical parts of the villi in rats receiving a standard diet containing the recommended level of Cu in the form of $CuCO_3$ and cellulose as a source of fibre. We observed no adverse changes in the morphology of the small intestine in rats receiving a diet containing the 6.5 mg/kg of CuNPs and the addition of cellulose (control group) or pectin. For rats fed a diet containing the same level of CuNPs but supplemented with inulin or psyllium, single tissue defects were observed at the top of the intestinal villi. Similar changes were intensified by the inclusion of a higher level of CuNPs (13 mg/kg) in the diet, regardless of the form of fibre used, with the most significant and numerous losses of the top part of the intestinal villi observed in the group receiving cellulose in the diet. The obtained results indicate that the replacement of $CuCO_3$ by CuNPs had a beneficial effect on the morphology of the intestines. In turn, changes in the top parts of the villi observed in groups of rats fed a diet containing an increased level of CuNPs, regardless of the fibre source used, seem to be related to the direct effect of Cu in the form of nanoparticles. However, the lack of any pathological changes at the base of the villi and in the mucosa of the small intestine, regardless of the type of dietary fibre, as well as the lack of deterioration of indicators proving the integrity of the intestinal barrier, do not seem to confirm this assumption. Moreover, our previous studies did not show any pathological changes in the small intestine tissue of healthy, normotensive rats, in which both 50% and 100% of the traditional $CuCO_3$ was replaced by CuNPs. In the histological image of the small intestine of rats receiving the recommended level of Cu in the diet only in the form of $CuCO_3$, isolated extensive changes in the base of intestinal villi were found [31]. In light of the above, it can be assumed that the observed damage to the top parts of the intestinal villi may be the result of a change in the rheology of the gastrointestinal content due to the presence of dietary fibre. Dietary fibre, binding water, increases its volume, and thus, the pressure of intestinal contents on the walls of the small intestine. It is therefore highly probable that the movement of the swollen, viscous chyme through successive fragments of the small intestine was accompanied by greater friction, thus leading to damage to the top parts of the intestinal villi.

Numerous reports in the literature indicate that the use of Cu nanoparticles in animal nutrition may be associated with many risks, such as their increased accumulation in internal organs [11,17]. Henson et al. [64], in an in vitro model study, showed that CuO nanoparticles are much more cytotoxic to rat intestinal epithelial cells (IEC-6) than Cu^{2+} ions, and this effect is stronger the higher their dose and the longer the exposure time. The authors observed that increased production of free oxygen radicals resulted in damage to the mitochondrial membrane and reduced viability of small intestine cells exposed to CuO NPs [64]. Lee et al. [17] suggest that the toxicity of copper nanoparticles may be a

result of the fact that, in the acidic conditions of gastric juice, they can dissociate to Cu^{2+} ions. However, the authors did not record a similar phenomenon in intestinal juice [17]. Henson et al. also confirmed that there was no release of Cu^{2+} ions from CuO NPs in the intestinal juice [64]. According to the authors, this may indicate that the cytotoxicity of CuO NPs towards intestinal cells is not dependent on their dissociation into the ionic form, but on the specific and natural nature of this form of Cu [64]. The results of our previous studies have also shown that CuNPs are absorbed in the small intestine to a much greater extent than standard forms of this element, such as $CuCO_3$ [11], which, in light of the results obtained by Lee et al. [17] and Henson et al. [64], should result in damage to the intestinal barrier and the occurrence of pathological changes in the examined tissue. The results of our study, apart from the possibility of intensifying DNA oxidation by CuNPs, did not confirm this assumption, indicating the highly toxic effect of CuNPs disrupting the integrity of the intestinal barrier, even when used in a dose two times higher than the nutritional recommendations for rats. The introduction of twice as high levels of CuNPs into the diet did not have a significant effect on the increased level of this element in the blood plasma (unpublished data). This effect may therefore be related to the direct effect of dietary fibre introduced into the diet. So far, it has not been possible to clearly determine the effect of different types of dietary fibre on the intestinal absorption of minerals. There are reports indicating both a beneficial and negative effect of dietary fibre [22–25]. According to Coudray [27], mineral absorption also largely depends on the type and amount of fibre in the diet, as well as mineral homeostasis in the body. Coudray et al. [25] showed an increase in the absorption and retention of Cu in the intestines as a result of inulin consumption. In turn, Krzysik et al. [26] observed a decrease in the absorption of divalent ions as a result of feeding rats a diet containing pectin or cellulose. The decrease in the absorption of trace elements in the small intestine is probably due to the fact that fibre (especially pectin and psyllium) forms a kind of sticky gel in the digestive tract, which is able to bind mainly divalent ions (including Cu^{2+}) with free carboxyl groups, which reduces their bioavailability [23,26,65]. Furthermore, pectin may also affect the absorption of minerals by stimulating the bacterial production of short-chain fatty acids (SCFA) and acidifying the intestinal lumen, thus creating unfavourable conditions for the absorption process and intensifying the dissociation of CuNPs to divalent ions [8,26,66]. In addition, the presence of dietary fibre in the diet accelerates the intestinal transit, as a result of which the food content is much shorter in the small intestine, which may significantly reduce the amount of nutrients and minerals absorbed into the body [26,67]. Taken together, it seems that the supplementation of dietary fibre in the form of pectin, inulin and psyllium reduced the absorption of CuNPs, thus protecting the small intestine and modulating its biological response.

5. Conclusions

To conclude, replacing $CuCO_3$ by CuNPs in the diet of rats positively reduced the level of lactic acid and markers of apoptotic cell death in the small intestine; however, it resulted in the intensification of DNA oxidation. Increasing the level of CuNPs from 6.5 to 13 mg Cu/kg of diet had no negative effect on the physiological intestinal response. Our results indicate that the most visible and beneficial effect on DNA repair mechanisms is related to inulin, while pectin has the greatest ability to inhibit inflammatory processes that induce apoptotic death of cells in the small intestine. The obtained results suggest that dietary fibre supplementation in rats' diet effectively protects the small intestine against potentially harmful, oxidative effects of CuNPs by intensifying the intestinal barrier, and this may finally translate into a beneficial regulation of their metabolic effect.

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Institutional Review Board Statement: The experimental protocol consisted of 10 groups, n = 10 per group. All animal care and experimental schema were in accordance with the Polish legislation acts concerning ani-mal experimentation and ethical committee according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, Directive 2010/63/EU and it was approved by the Local Ethics Committee for Animal Experiments in Olsztyn Local Animal Care and Use Committee (Approval No. 19/2021; Olsztyn, Poland). The study was carried out in compliance with the ARRIVE guidelines. Every effort was made to minimise the suffering of the animals used in the experiment.

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The Effect of Copper Nanoparticles on Liver Metabolism Depends on the Type of Dietary Fiber

Aleksandra Marzec [1], Bartosz Fotschki [2,*], Dorota Napiórkowska [2], Joanna Fotschki [2], Ewelina Cholewińska [1], Piotr Listos [3], Jerzy Juśkiewicz [2] and Katarzyna Ognik [1]

[1] Department of Biochemistry and Toxicology, Faculty of Animal Sciences and Bioeconomy, University of Life Sciences in Lublin, Akademicka 13, 20-950 Lublin, Poland; aleksandra.marzec@up.lublin.pl (A.M.); ewelina.cholewinska@up.lublin.pl (E.C.); katarzyna.ognik@up.lublin.pl (K.O.)

[2] Division of Food Science, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Tuwima 10, 10-748 Olsztyn, Poland; d.napiorkowska@pan.olsztyn.pl (D.N.); j.fotschki@pan.olsztyn.pl (J.F.); j.juskiewicz@pan.olsztyn.pl (J.J.)

[3] Sub-Department of Pathomorphology and Forensic Medicine, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, Głęboka 30, 20-612 Lublin, Poland; piotr.listos@up.lublin.pl

* Correspondence: b.fotschki@pan.olsztyn.pl

Abstract: Background/Objectives: A diet enriched with copper nanoparticles (CuNPs) exhibits a wide range of effects on liver metabolism, both positive and negative. Dietary fibers are the key components that may affect the absorption of minerals, including copper, and change their impact on organisms. Methods: Therefore, this study investigated whether and how supplementation with different sources of dietary fiber (cellulose, pectin, inulin, and psyllium) affects the function of CuNPs in the liver of male Wistar rats. Results: The results showed that CuNPs at different doses had varying effects on lipid metabolism and inflammation in the liver. Specifically, higher doses of CuNPs were associated with increased lipid accumulation and the activation of pro-inflammatory mechanisms. However, combining CuNPs with dietary fibers, such as psyllium and inulin, was beneficial in mitigating the effects of the examined nanoparticles, leading to reduced fat, cholesterol, and triglycerides in the liver. Combining psyllium with CuNPs showed the most substantial effect on liver metabolism and inflammation parameters. Furthermore, hepatic histology analyses showed that adding psyllium to the diet with CuNPs reduces changes associated with fat accumulation and mononuclear cell infiltration. The observed beneficial changes in the liver may have been related to a reduction in the gene expression level of sterol regulatory element-binding protein 1 and peroxisome proliferator-activated receptor gamma and cyclooxygenase-2. Conclusions: In conclusion, enriching the diet with dietary fibers such as psyllium can regulate the action of CuNPs, thereby improving lipid metabolism and reducing inflammation in the liver.

Keywords: copper nanoparticles; psyllium; dietary fiber; triglycerides; liver metabolism; aminotransferases

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1. Introduction

Due to their small size and high surface-to-volume ratio, copper nanoparticles (CuNPs) exhibit unique properties that make them useful in various applications, including antimicrobial agents, electronics, and agriculture [1–3]. Another way to use these nano molecules is as an ingredient in food products. These compounds show a wide range of effects on the consumer's body, including both positive and negative effects [4].

The liver plays a crucial role in processing compounds absorbed from the digestive tract, making CuNPs in the diet a vital component, with the potential to influence liver metabolism [5]. Using CuNPs as a therapeutic agent for liver metabolic disorders presents promising opportunities as well as significant risks. CuNPs have shown potential in treating non-alcoholic fatty liver disease (NAFLD) by scavenging reactive oxygen species (ROS) and reducing oxidative stress, which are critical factors in the progression of liver

diseases [6,7]. Specifically, ultrasmall copper-based nanoparticles combined with resveratrol have demonstrated efficacy in targeting liver tissues and treating inflammation in NAFLD, suggesting a novel approach for managing such conditions [6]. Other in vitro studies have shown the potential of CuNPs as anticancer agents, particularly in targeting colorectal cancer cells [8]. Additionally, the low-dose administration of various inorganic nanoparticles, including CuNPs, has been found to promote lipid degradation and alleviate liver steatosis without causing adverse effects, indicating a potential therapeutic benefit for metabolic regulation [9]. Moreover, the study involving obese individuals with liver steatosis showed that the level of copper in serum played a significant role in predicting the onset of atherosclerosis [10]. However, the hepatotoxicity of CuNPs cannot be overlooked. Studies have shown that high doses of CuNPs can induce significant oxidative stress, inflammation, and histopathological changes in the liver, leading to severe hepatic damage and impaired drug metabolism [11,12]. Furthermore, repeated exposure to CuNPs has been associated with profibrotic changes and immunosuppressive effects [13]. Recent nutritional studies on mice have shown that copper deficiency is strongly associated with metabolic dysfunction-associated steatotic liver disease (MASLD), and CuNPs can exacerbate this condition by disrupting copper metabolism and inducing oxidative stress [14]. The size of the nanoparticles also plays a crucial role, with smaller nanoparticles showing reversible toxicity and better clearance compared to larger ones, which tend to accumulate in the liver and cause prolonged damage [15].

One effective method to modulate the absorption of CuNPs and their impact on the body might be to enhance the diet with fiber. This dietary component can impact the bioavailability of CuNPs through various mechanisms, including mineral binding, fermentation, and interaction with other dietary components. Dietary fibers can bind minerals and form complexes that are difficult to absorb in the intestinal lumen, potentially decreasing mineral bioavailability [16]. However, the type of dietary fiber plays a crucial role in this interaction. For instance, inulin has been shown to have a beneficial effect on DNA repair mechanisms. At the same time, pectin can inhibit inflammatory processes in the small intestine, thereby protecting against the oxidative effects of CuNPs [17]. The physicochemical properties of fibers, such as solubility and fermentability, also influence their interaction with minerals. Soluble fibers are rapidly fermented, which can liberate bound minerals and promote colonic absorption, potentially offsetting any negative effects on mineral bioavailability [18]. Additionally, the interaction between dietary fibers and copper ions can be complex, as demonstrated by the use of Electron Paramagnetic Resonance (EPR) techniques to study barley β-glucan and copper ions, revealing weak interactions under physiological conditions [19].

Previous nutritional research has shown the protective role of dietary fiber supplementation against the potentially harmful oxidative effects of CuNPs in the small intestine [17]. Furthermore, the dietary combination with dietary fiber affects the absorption of CuNPs from the gastrointestinal tract, thus modulating their levels in the liver [20]. However, there is no information on how the type of dietary fiber modulates the effect of CuNPs on liver function. Therefore, the main objective of this study was to evaluate the dosage of CuPNs and dietary combinations of four different sources of dietary fiber (cellulose, pectin, inulin, and psyllium) with CuPNs on the metabolism and inflammation in the liver of rats. As far as animal models are concerned, there is considerable similarity between rodent and human internal organs, including liver functioning. Although in vivo data on metal nanoparticles obtained from rats may not be entirely transferred to humans, it must be stressed that such new information pushes our knowledge forward.

2. Materials and Methods

The protocol containing the research questions, experimental schema with the in vivo study design, and analysis plan was submitted to the reviewers of the National Science Center (Kraków, Poland) for evaluation, and was subsequently approved and funded.

2.1. Dietary Fibres and Copper Nanoparticles

The copper nanoparticles (99.9% purity) were acquired from SkySpring Nanomaterials, Inc. located in Houston, TX, USA. The detailed physicochemical properties were described by Cholewińska et al. (2023) [17]. For the control dietary sources, $CuCO_3$ was purchased from Merck KGaA in Darmstadt, Germany, and α-cellulose from Sigma in Poznań, Poland. Experimental dietary fiber sources such as pectin (PectinE 440(I) from Brouwland in Beverlo, Belgium), inulin (Frutafit Tex from Sensus in Roosendaal, the Netherlands), and psyllium (Psyllium husk powder from NaturaleBio in Rome, Italy) were also used to design the experimental diets.

2.2. In Vivo Experiment

A total of 100 nine-week-old healthy outbred male Wistar rats (Cmdb:Wi) were part of the research study. The animals were originated from the Medical University of Bialystok, Poland. After a two-week acclimatization period, they were randomly divided into ten groups, with each group consisting of ten rats. Random numbers were created using Microsoft Excel's standard = RAND() function (version 15.0.5589.1000). The rats were housed individually in metabolic cages in a carefully controlled environment. The environment included a 12 h light–dark cycle, a temperature maintained at 21 ± 1 °C, a relative humidity ranging from 50% to 70%, and 20 air changes per hour. Throughout a six-week period, each group was given tap water and a modified version of the semipurified casein diet, as recommended for laboratory rodents by the American Institute of Nutrition. The experimental diets in this study consisted of two levels of copper nanoparticles (CuNPs); the recommended level and the double that level were 6.5 and 13 mg/kg, respectively. These levels of CuNPs were paired with various types of dietary fiber. The control diet included a mineral mixture containing standard and high levels of $CuCO_3$ (6.5 and 13 mg/kg diet). In contrast, the diets with CuNPs utilized a mineral mixture without $CuCO_3$. The control dietary fiber, α-cellulose, was incorporated at 8% of the diet, while the experimental fibers, including 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 CuNPs were administered in oil to prevent oxidation, and the experimental diet was kept in the freezer (−70 °C) before administration. The diets provided to the rats throughout the entire experimental period consisted of a detailed composition, which can be found in Table 1. The animals were given unrestricted access to these diets. All animal procedures strictly followed the guidelines outlined in the European Union Directive (2010/63/EU) for animal experiments. Furthermore, the experiment received approval from the local Institutional Animal Care and Use Committee under Permission No. 19/2021 (17 March 2021) in Olsztyn, Poland. The study was conducted in accordance with the ARRIVE guidelines, and all possible measures were taken to minimize the suffering of the animals used in the experiment. During the period of experimental feeding, in the event of adverse effects related to humane endpoints, i.e., cessation of diet intake for more than 2 days, making specific sounds as a pain signal for more than 1 h, the appearance of neurological symptoms (e.g., ataxia, impairment in maintaining a favorable body position), and the presence of blood in the feces for more than 1 day, a veterinarian (employed for these purposes at the institute) could make a decision of humane euthanasia, using the method of gradually filling the chamber of the animal with carbon dioxide or the method of dislodging the cervical vertebrae of a previously sedated animal. To the best of our knowledge, based on literature and our previous experiments, the above symptoms should not be related to the experimental factors (fiber, nanoparticles) and there is only a minimal risk of their occurrence.

Table 1. The experimental diets given to the rats for six 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
Other components [3]	77.0	77.0	77.0	77.0	77.0	77.0	77.0	77.0	77.0	77.0
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

C, control diet with a standard Cu content in the mineral mix and 8% of cellulose as a dietary fiber source; CH, control diet with an enhanced Cu content in the mineral mix and 8% of cellulose as a dietary fiber; CN, diet with a standard CuNPs content in the mineral mix and 8% of cellulose as a dietary fiber source; CNH, diet with enhanced CuNPs content in the mineral mix and 8% of cellulose as a dietary fiber; PN, diet with a standard CuNPs content in the mineral mix and 2% of cellulose and 6% of pectin as a dietary fiber source; PNH, diet with enhanced CuNPs content in the mineral mix and 2% of cellulose and 6% of pectin as a dietary fiber source; JN, diet with a standard CuNPs content in the mineral mix and 2% of cellulose and 6% of inulin as a dietary fiber source; JNH, diet with enhanced CuNPs content in the mineral mix and 2% of cellulose and 6% of inulin as a dietary fiber source; SN, diet with a standard CuNPs content in the mineral mix and 2% of cellulose and 6% of psyllium as a dietary fiber source; SNH, diet with enhanced CuNPs content in the mineral mix and 2% of cellulose and 6% of psyllium as a dietary fiber source. [1] Casein preparation: crude protein 89.7%, crude fat 0.3%, ash 2.0%, and water 8.0%. [2] α-Cellulose (SIGMA, Poznan, Poland), main source of dietary fiber. [3] 0.2% choline chloride, 8.0% rapeseed oil, 64.0% maize starch, 1.0% vitamin mix (AIN-93G-VM), and 3.5% mineral mix were added to each diet. In the experimental treatments involving copper nanoparticles (CuNPs), the mineral mix was intentionally devoid of copper carbonate ($CuCO_3$). To ensure the safety of the operator during the preparation of the experimental diets, the CuNPs preparation was incorporated into an emulsion, along with dietary rapeseed oil. This approach, which has been successfully employed in the previous study, helped to mitigate potential risks associated with handling CuNPs and ensured the effective administration of the treatment.

2.3. Collection of Biological Material and Analytical Procedures

None of the animals were excluded from the experiment. The project manager was the only person who was aware of the animals' allocation to a particular study group. None of the analysis contractors were acquainted with the treatment animal allocation. During the experiment, the rats were monitored for feed intake and body weight (data published in Cholewińska et al. 2023) [17]. After a period of 6 weeks on the experimental diet, the rats underwent anesthesia with a combination of ketamine and xylazine (100 mg and 10 mg/kg BW, respectively). Subsequently, blood samples were collected from the vena cava into heparinized tubes and then centrifuged at a low speed for 10 min at $350\times g$ and 4 °C. The resulting plasma samples were stored at −70 °C until analysis. Following this, the liver was extracted, weighed, and rapidly frozen using liquid nitrogen. Lipids were then extracted from the liver using the Folch et al. (1957) [21] method. A total of 0.2 g of tissue was used for extraction. The extraction process involved using a mixture of chloroform and methanol in a 2/1 ratio. A total of 4 mL of the mixture was added to the tissue and then homogenized. After centrifugation, the chloroform layer was collected, and the solvent was evaporated under nitrogen. The resulting sample was then used for further determinations. The concentrations of liver cholesterol and triglycerides were determined using spectrophotometric techniques with commercial kits (Cholesterol DST, Triglycerides DST, Alpha Diagnostics, Ltd., San Antonio, TX, USA). The sample size was determined for each analysis based on previous research.

2.4. Blood Plasma Parameters of Metabolism and Inflammation

The plasma concentration of cholesterol (total and its HDL), triglycerides, creatinine, uric acid, urea, glucose, and the plasma activities of aspartate transaminase (AST) and alanine transaminase (ALT) were determined using an automatic biochemical analyzer (Pentra C200, Horiba, Tokyo, Japan). The atherogenic index of plasma was calculated according to the following formula: log(TG/HDL). To measure the concentration of insulin, glucagon, and ghrelin in the plasma, validated rat ELISA kits were used (Shanghai Qayee Biotechnology Co. Ltd., Shanghai, China).

2.5. Liver Gene Expression

Firstly, RNA was extracted from the liver using a TRI Reagent solution (Thermo Fisher Scientific, Waltham, MA, USA), as per the manufacturer's instructions. *β-actin* was chosen as the reference gene. The mRNA expression levels of peroxisome proliferator-activated receptor gamma (*ppar-γ*, Cat# Rn00440945_m1), peroxisome proliferator-activated receptor alpha (*ppar-α*, Cat# Rn00566193_m1), sterol regulatory element-binding protein 1 (*srebp1c*, Cat# Rn01495769_m1), and cyclooxygenase-2 (*cox-2*, Cat# Rn01483828_m1) were measured using single tube TaqMan® Gene Expression Assays (Life Technologies, Carlsbad, CA, USA). Amplification was carried out using a 7900HT Fast Real-Time PCR System under the following conditions: initial denaturation for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Finally, the mRNA expression levels of the selected genes were normalized to *β-actin* (Cat# Rn00667869_m1).

2.6. Liver Histopathology

Histopathological examinations, including hematoxylin eosin staining of liver samples from rats, were conducted following the procedure described in our previous study [22].

2.7. Statistical Analysis

The differences among treatment groups were determined using STATISTICA software, version 12.0 (StatSoft Corp., Krakow, Poland). A two-way analysis of variance (ANOVA) was used to evaluate the effects of two main factors, the dosage of copper nanoparticles (CuNPs) (low dose, 6.5 mg/kg and high dose, 13 mg/kg) and the type of dietary fiber (cellulose, pectin, inulin, and psyllium). Following the ANOVA, Duncan's multiple range test was conducted. The data were checked for normality with the aid of the Shapiro–Wilk test. Furthermore, to compare each experimental group fed the low dose of CuNPs with the control group (fed a diet with 6.5 mg/kg Cu from $CuCO_3$ and containing cellulose as the primary dietary fiber source), a *t*-test was employed. Similarly, the *t*-test was used to compare the experimental groups fed diets with the high dose of CuNPs with the control group, CH, in which rats were fed a diet with 13 mg/kg Cu from $CuCO_3$ and containing cellulose as the primary dietary fiber source. Differences with a significance level of $p \leq 0.05$ were considered to be statistically significant.

3. Results

The basic parameters of growth and dietary intake shown in previous work [17] indicate that the experimental diets had no effect on the final weight and body weight gain in rats, while dietary intake significantly decreased when inulin and psyllium were added to the diets with CuNPs. The relative weight of the liver was the highest in rats fed diets with inulin ($p < 0.05$ vs. the treatment with psyllium), regardless of the CuNPs dose (Table 2). The D × F interaction showed the highest fat content was observed in the hepatic tissue in the CHH and PN groups ($p < 0.05$ vs. all other groups), while the lowest fat content was noted in the SNH rats ($p < 0.05$ vs. remaining groups except SN and JN). Irrespective of the nanoparticle dose, psyllium treatment decreased hepatic total cholesterol and triglycerides concentration, as well as the activity of plasma ALT, as compared to other treatments with cellulose, pectin, or inulin. Additionally, we observed reduced ALT activity in the pectin treatment in comparison to the cellulose one ($p < 0.05$). The CuNPs dose by

fiber interaction showed the highest plasma AST activity occurred in the CNH and PNH rats ($p < 0.05$ vs. remaining groups except CN), while the lowest AST was noted in the JN and JNH groups ($p < 0.05$ vs. CN, CNH, PNH).

Table 2. Liver function indicators and the levels of AST and ALT in the blood plasma of rats that were fed experimental diets ($n = 10$ per group) *.

	Weight g/100 g BW	**Fat %**	**TC mg/g**	**TG mg/g**	**AST U/L**	**ALT U/L**
Control C	2.80 ± 0.04	14.8 ± 0.74	10.9 ± 1.03	13.8 ± 1.08	57.5 ± 1.55	17.6 ± 0.93
Control CH	2.83 ± 0.07	13.8 ± 0.97	10.8 ± 0.68	13.3 ± 0.93	59.6 ± 1.14	19.0 ± 0.99
Two-way ANOVA:						
CN	2.83 ± 0.08	12.2 ± 0.96 $^{b\#}$	6.42 ± 0.58 $^{\#}$	10.8 ± 0.48 $^{\#}$	63.1 ± 0.67 $^{ab\#}$	19.1 ± 0.66
CNH	2.78 ± 0.06	18.8 ± 1.39 $^{a\&}$	8.71 ± 1.04	11.1 ± 1.15	66.7 ± 1.88 $^{a\&}$	21.8 ± 1.10
PN	2.67 ± 0.04 $^{\#}$	18.3 ± 1.13 $^{a\#}$	6.40 ± 0.79 $^{\#}$	11.4 ± 1.07	59.2 ± 1.82 ^{bc}	18.2 ± 0.83
PNH	2.73 ± 0.06	12.5 ± 0.81 ^{b}	7.11 ± 0.88 $^{\&}$	12.8 ± 0.98	68.1 ± 2.07 $^{a\&}$	18.5 ± 0.45
JN	2.78 ± 0.07	8.26 ± 0.85 $^{cd\#}$	8.25 ± 1.09	10.8 ± 0.98 $^{\#}$	55.7 ± 1.65 ^{c}	19.1 ± 0.96
JNH	2.93 ± 0.18	8.49 ± 0.84 $^{c\&}$	7.43 ± 0.47 $^{\&}$	10.4 ± 0.85 $^{\&}$	55.5 ± 1.88 ^{c}	19.3 ± 0.93
SN	2.58 ± 0.06 $^{\#}$	7.90 ± 0.57 $^{cd\#}$	3.67 ± 0.23 $^{\#}$	9.11 ± 0.56 $^{\#}$	60.0 ± 1.91 ^{bc}	16.6 ± 0.78
SNH	2.65 ± 0.05	5.56 ± 067 $^{d\&}$	2.97 ± 0.55 $^{\&}$	6.87 ± 0.57 $^{\&}$	60.1 ± 1.91 ^{bc}	16.4 ± 0.73 $^{\&}$
SEM	0.026	0.511	0.344	0.333	0.654	0.297
CuNPs dose (D)						
L (6.5 mg/kg)	2.72	11.6	6.18	10.5	59.5	18.2
H (13 mg/kg)	2.76	11.4	6.55	10.3	62.3	18.9
p value	0.509	0.656	0.487	0.697	0.025	0.243
Fiber type (F)						
C (cellulose)	2.78 ^{ab}	15.5	7.56 ^{a}	11.0 ^{a}	64.9	20.5 ^{a}
P (pectin)	2.70 ^{ab}	15.4	6.76 ^{a}	12.1 ^{a}	63.7	18.3 ^{b}
J (inulin)	2.85 ^{a}	8.38	7.84 ^{a}	10.6 ^{a}	55.0	19.1 ^{ab}
S (psyllium)	2.62 ^{b}	6.73	3.32 ^{b}	7.99 ^{b}	60.0	16.5 ^{c}
p value	0.041	<0.001	<0.001	<0.001	<0.001	<0.001
Interaction D × F						
p value	0.504	<0.001	0.146	0.212	0.022	0.256

* The experimental feeding period involved different dietary treatments: groups C, control diet with a standard Cu content in the mineral mix and 8% of cellulose as a dietary fiber source; group CH, control diet with an enhanced Cu content in the mineral mix and 8% of cellulose as a dietary fiber; group CN, diet with a standard CuNPs content in the mineral mix and 8% of cellulose as a dietary fiber source; group CNH, diet with enhanced CuNPs content in the mineral mix and 8% of cellulose as a dietary fiber; group PN, diet with a standard CuNPs content in the mineral mix and 2% of cellulose and 6% of pectin as a dietary fiber source; group PNH, diet with enhanced CuNPs content in the mineral mix and 2% of cellulose and 6% of pectin as a dietary fiber source; group JN, diet with a standard CuNPs content in the mineral mix and 2% of cellulose and 6% of inulin as a dietary fiber source; group JNH, diet with enhanced CuNPs content in the mineral mix and 2% of cellulose and 6% of inulin as a dietary fiber source; group SN, diet with a standard CuNPs content in the mineral mix and 2% of cellulose and 6% of psyllium as a dietary fiber source; group SNH, diet with enhanced CuNPs content in the mineral mix and 2% of cellulose and 6% of psyllium as a dietary fiber source. Mean values within a column with different superscript letters differ significantly ($p < 0.05$). Differences among the groups (CN, CNH, PN, PNH, JN, JNH, SN, SNH) are indicated with superscripts only if there is a statistically significant interaction D × F ($p < 0.05$). Each experimental group fed CuNPs 6.5 mg/kg (CN, PN, JN, SN) was compared with the control C group using a *t*-test (# indicates a significant difference versus the C group). Similarly, each experimental group fed CuNPs 13 mg/kg (CNH, PNH, JNH, SNH) was compared with the control CH group using a *t*-test (& indicates a significant difference versus the CH group). The results are presented as the mean ± standard error of the mean (SEM). ALT, alanine aminotransferase; AST, aspartate aminotransferase; TC, total cholesterol; TG, triglycerides.

The *t*-test revealed a diminished relative liver weight in the PN and SN rats compared to the C control ($p < 0.05$). Hepatic fat concentration was significantly enhanced in the CNH and PN groups vs. their respective controls, i.e., the CH and C, respectively (*t*-test; $p < 0.05$). In the remaining groups fed diets with copper nanoparticles, except PNH, the liver fat concentration was decreased in comparison to the respective controls fed diets with $CuCO_3$ (*t*-test; $p < 0.05$). As compared to the respective controls, the hepatic total cholesterol was diminished in all experimental groups with CuNPs, except CNH and JN. In the case of

hepatic triglycerides, such decreases were noted in the CN, JN, JHNH, SN, and SNH rats ($p < 0.05$; *t*-test). The *t*-test showed significantly enhanced blood plasma AST activity in the CN (vs. C), CNH (vs. CH), and PNH (vs. CH) groups. The plasma ALT activity was lower in SNH rats than CH ones (*t*-test; $p < 0.05$).

The two-way ANOVA showed that irrespective of the CuNPs dose, the treatments with functional fiber, i.e., pectin, inulin, and psyllium, caused a significant increase in the plasma HDL concentration, thus decreasing the TC/HDL ratio compared to the cellulose treatment (Table 3). The lowest AIP index value was noted in the psyllium treatment, regardless of the CuNPs dose ($p < 0.05$ vs. C and P treatments). Additionally, the AIP noted in the inulin treatment was lower than in the C one ($p < 0.05$). The *t*-test showed that in comparison to a respective control, the SNH rats had decreased creatinine concentrations, and the PNH and SN animals had decreased uric acid levels in their blood plasma. As compared to the C control, plasma urea was significantly enhanced in the JN group (*t*-test). The plasma HDL concentration was enhanced in the PN and SN rats (vs. control C; *t*-test), as well as in the JNH and SNH animals (vs. control CH; *t*-test). The TC/HDL ratio decreased in the PN, JN, JNH, SN, and SNH rats in comparison to their respective controls ($p < 0.05$; *t*-test). The AIP index decreased in rats JNH vs. CH ($p < 0.05$; *t*-test).

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

	Creat. µmol/L	UA µmol/L	Urea mmol/L	HDL mmol/L	TC mmol/L	TG mmol/L	GL mmol/L	AIP	TC/HDL
Control C	23.6 ± 0.95	35.4 ± 1.85	3.99 ± 0.22	0.397 ± 0.026	1.58 ± 0.09	0.927 ± 0.08	12.4 ± 0.63	0.361 ± 0.049	4.04 ± 0.18
Control CH	24.0 ± 0.72	34.2 ± 2.28	4.20 ± 0.09	0.417 ± 0.022	1.69 ± 0.08	1.05 ± 0.07	13.9 ± 0.71	0.399 ± 0.033	4.08 ± 0.13
Two-way ANOVA:									
CN	25.0 ± 1.03	33.4 ± 1.82	4.20 ± 0.11	0.442 ± 0.023	1.75 ± 0.08	1.22 ± 0.12	13.3 ± 0.85	0.427 ± 0.045	4.01 ± 0.16
CNH	23.5 ± 0.71	34.8 ± 2.54	4.35 ± 0.17	0.417 ± 0.030	1.66 ± 0.08	1.01 ± 0.05	13.8 ± 0.57	0.387 ± 0.039	4.06 ± 0.19
PN	22.7 ± 0.95	29.7 ± 2.59	4.28 ± 0.09	0.491 ± 0.022 #	1.64 ± 0.07	1.16 ± 0.08	13.7 ± 0.50	0.367 ± 0.034	3.35 ± 0.08 #
PNH	22.7 ± 0.91	28.2 ± 1.60 &	4.39 ± 0.15	0.467 ± 0.027	1.66 ± 0.08	1.16 ± 0.10	13.7 ± 0.42	0.389 ± 0.041	3.61 ± 0.18
JN	22.5 ± 0.89	30.1 ± 2.56	4.84 ± 0.19 #	0.462 ± 0.031	1.56 ± 0.07	0.901 ± 0.08	13.2 ± 0.76	0.283 ± 0.046	3.46 ± 0.19 #
JNH	23.7 ± 1.00	33.2 ± 3.15	4.57 ± 0.20	0.499 ± 0.019 &	1.61 ± 0.09	0.960 ± 0.05	14.2 ± 0.63	0.283 ± 0.026 &	3.26 ± 0.23 &
SN	23.3 ± 1.63	28.9 ± 1.96 #	4.56 ± 0.33	0.499 ± 0.022 #	1.67 ± 0.09	0.926 ± 0.14	12.4 ± 0.37	0.200 ± 0.100	3.35 ± 0.14 #
SNH	21.8 ± 0.74 &	29.5 ± 2.22	4.28 ± 0.17	0.504 ± 0.019 &	1.73 ± 0.08	1.12 ± 0.12	12.7 ± 0.64	0.326 ± 0.046	3.44 ± 0.10 &
SEM	0.309	0.740	0.060	0.008	0.025	0.030	0.198	0.016	0.059
CuNPs dose (D)									
L (6.5 mg/kg)	23.4	30.5	4.47	0.474	1.66	1.05	13.1	0.319	3.54
H (13 mg/kg)	22.7	31.5	4.42	0.472	1.67	1.06	13.6	0.346	3.60
p value	0.382	0.548	0.692	0.932	0.841	0.864	0.294	0.470	0.622
Fibre type (F)									
C (cellulose)	24.2	34.1	4.27	0.430 [b]	1.70	1.11	13.5	0.407 [a]	4.04 [a]
P (pectin)	22.7	29.0	4.33	0.479 [a]	1.65	1.16	13.7	0.378 [ab]	3.48 [b]
J (inulin)	22.8	31.9	4.75	0.481 [a]	1.59	0.933	13.7	0.284 [bc]	3.37 [b]
S (psyllium)	22.5	29.2	4.42	0.502 [a]	1.70	1.02	12.6	0.263 [c]	3.40 [b]
p value	0.285	0.105	0.070	0.033	0.511	0.112	0.221	0.017	<0.001
Interaction D × F									
p value	0.687	0.766	0.594	0.551	0.719	0.232	0.931	0.435	0.662

* Description same as in Table 2. The results are presented as the mean ± standard error of the mean (SEM). Mean values within a column with different superscript letters differ significantly ($p < 0.05$). Differences among the groups (CN, CNH, PN, PNH, JN, JNH, SN, SNH) are indicated with superscripts only if there is a statistically significant interaction D × F ($p < 0.05$). Each experimental group fed CuNPs 6.5 mg/kg (CN, PN, JN, SN) was compared with the control C group using a *t*-test (# indicates a significant difference versus the C group). Similarly, each experimental group fed CuNPs 13 mg/kg (CNH, PNH, JNH, SNH) was compared with the control CH group using a *t*-test (& indicates a significant difference versus the CH group). TC, total cholesterol (mmol/L); HDL, high density lipoprotein (mmol/L); TG, triglycerides (mmol/L); AIP, atherogenic index of plasma [log(TG/HDL)]; UA, uric acid (µmol/L); Urea, mmol/L; Creat., creatinine (µmol/L); GL, glucose (mmol/L).

A significant CuNPs by fiber interaction showed that insulin concentration in the plasma of rats fed diets PN, PNH, JN, and SNH was enhanced as compared to JNH rats ($p < 0.05$; Table 4). The highest and lowest plasma ghrelin concentration was noted in the JNH and SNH groups, respectively (in both cases $p < 0.05$ vs. remaining groups). The highest glucagon concentration in the plasma followed feeding with diet CN ($p < 0.05$ vs. all other groups, except CNH), while the lowest glucagon levels were noted in the PN,

JNH, SN, and SNH rats ($p < 0.05$ vs. other groups; see significant D×F interaction). The *t*-test revealed that all groups fed diets with copper nanoparticles were characterized by diminished plasma insulin levels when compared to their respective controls ($p < 0.05$ vs. C or CH). The ghrelin concentration in the plasma was enhanced in JNH rats vs. CH rats, while in the SNH rats, ghrelin levels were lower compared to the CH control ($p < 0.05$; *t*-test). The glucagon content in the plasma was decreased in the groups fed diets with copper nanoparticles, except CN, CNH, and PNH in comparison to the respective controls without CuNPs ($p < 0.05$; *t*-test).

Table 4. Blood levels of insulin, ghrelin, and glucagon in rats fed experimental diets.(n = 10 per group) *.

	Insulin µIU/mL	**Ghrelin pg/mL**	**Glucagon ng/mL**
Control C	16.6 ± 0.71	30.4 ± 1.46	9.99 ± 0.36
Control CH	15.6 ± 0.74	28.7 ± 1.61	8.37 ± 0.58
Two-way ANOVA:			
CN	10.7 ± 0.92 $^{ab\#}$	30.0 ± 1.94 ^{b}	9.70 ± 0.92 ^{a}
CNH	10.6 ± 0.30 $^{ab\&}$	29.2 ± 1.78 ^{b}	9.48 ± 0.51 ^{ab}
PN	11.6 ± 0.46 $^{a\#}$	29.5 ± 1.05 ^{b}	5.53 ± 0.38 $^{d\#}$
PNH	12.1 ± 0.72 $^{a\&}$	30.6 ± 1.18 ^{b}	7.07 ± 0.60 ^{c}
JN	12.0 ± 0.50 $^{a\#}$	30.7 ± 2.08 ^{b}	8.03 ± 0.31 $^{bc\#}$
JNH	9.71 ± 0.52 $^{b\&}$	35.1 ± 0.64 $^{a\&}$	5.52 ± 0.50 $^{d\&}$
SN	10.9 ± 0.41 $^{ab\#}$	29.9 ± 1.46 ^{b}	5.23 ± 0.44 $^{d\#}$
SNH	11.7 ± 0.52 $^{a\&}$	20.4 ± 1.15 $^{c\&}$	4.99 ± 0.33 $^{d\&}$
SEM	0.279	0.568	0.246
CuNPs dose (D)			
L (6.5 mg/kg)	11.3	30.0	7.12
H (13 mg/kg)	11.0	28.8	6.76
p value	0.489	0.258	0.342
Fiber type (F)			
C (cellulose)	10.7	29.6	9.59
P (pectin)	11.8	30.0	6.30
J (inulin)	10.8	32.9	6.78
S (psyllium)	11.3	25.2	5.11
p value	0.181	<0.001	<0.001
Interaction D × F			
p value	0.042	<0.001	0.004

* Description same as in Table 2. The results are presented as the mean ± standard error of the mean (SEM). Mean values within a column with different superscript letters differ significantly ($p < 0.05$). Differences among the groups (CN, CNH, PN, PNH, JN, JNH, SN, SNH) are indicated with superscripts only if there is a statistically significant interaction D × F ($p < 0.05$). Each experimental group fed CuNPs 6.5 mg/kg (CN, PN, JN, SN) was compared with the control C group using a *t*-test (# indicates a significant difference versus the C group). Similarly, each experimental group fed CuNPs 13 mg/kg (CNH, PNH, JNH, SNH) was compared with the control CH group using a *t*-test (& indicates a significant difference versus the CH group).

The hepatic *srebp-1c* expression was significantly enhanced in the CNH group, while the lowest expression of *srebp-1c* in the liver was observed in the SNH rats (Figure 1; in both cases $p < 0.05$ vs. all other groups). The CNH rats were also characterized by the highest hepatic *cox-2* expression ($p < 0.05$ vs. PN, JN, SN, SNH). The SNH liver had decreased *ppar-γ* expression as compared to other groups ($p < 0.05$). The *t*-test showed a decrease in *srebp-1c* and *ppar-γ* hepatic expression in SNH rats vs. control CH ($p < 0.05$). The *cox-2* expression was significantly higher in the CNH group than in the CH one (*t*-test).

The histopathological examination revealed minor changes in the liver structure of rats from the C and CH groups (Figure 2). A physiological structure with small clusters of mononuclear cell infiltration and slight congestion of the liver was observed in the CH group. The CN group showed a normal liver structure without any pathological changes. In the SN group, minor histological changes were observed, including minor fatty

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The histopathological examination revealed minor changes in the liver structure of rats from the C and CH groups (Figure 2). A physiological structure with small clusters of mononuclear cell infiltration and slight congestion of the liver was observed in the CH group. The CN group showed a normal liver structure without any pathological changes. In the SN group, minor histological changes were observed, including minor fatty changes and mononuclear cell infiltration. Additionally, the SNH, JN, and JNH groups, which were administered with inulin and psyllium, exhibited mild fatty degeneration and single

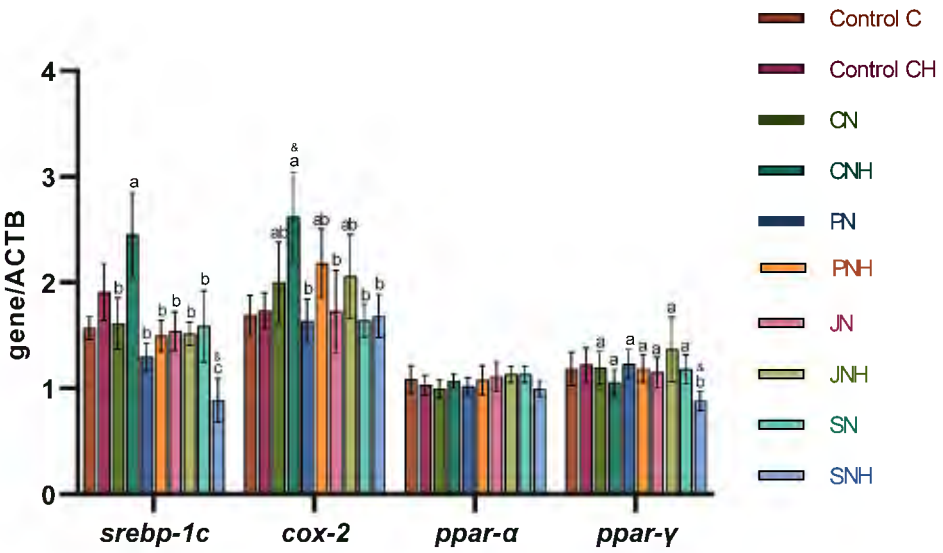

Figure 1. Hepatic mRNA expression (normalized to β-actin) of metabolic and inflammatory factors in rats fed experimental diets (n = 10 per group). Description of the groups same as in Table 2. *cox-2*, cyclooxygenase-2; *ppar-α*, peroxisome proliferator-activated receptor alpha; *ppar-γ*, peroxisome proliferator-activated receptor gamma; *srebp-1c*, sterol regulatory element-binding protein 1. Mean values within a column with different superscript letters differ significantly ($p < 0.05$). Similarly, each experimental group (CuNPs 13 mg/kg (CNH, PNH, JNH, SNH)) was compared with the control CH group using a *t*-test (& indicates a significant difference versus the CH group).

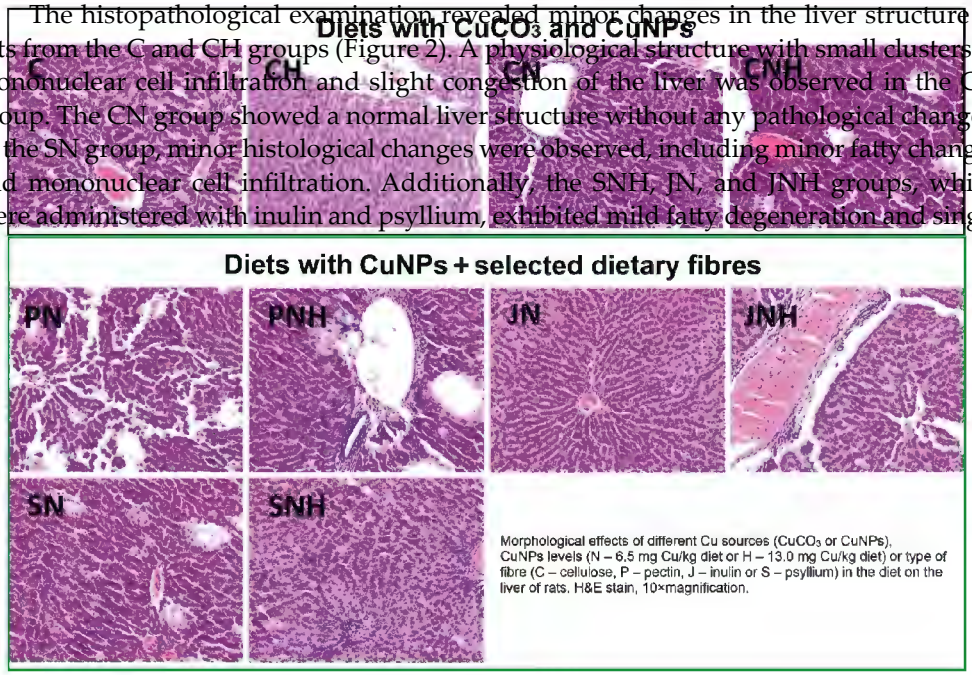

Figure 2. Hepatic histology in rats fed experimental diets. Description of the groups same as in Table 2. C, single foci of fatty degeneration of a physiological nature combined with the presence of small clusters of mononuclear cell infiltration; CH, physiological structure combined with slight congestion of the liver; CN, physiological structure of the liver and the presence of multiple small foci of fatty degeneration; CNH, extensive multiple-multifocal foci of fatty degeneration of the liver; PN, extensive singular fatty degeneration of the liver; PNH, extensive multifocal fatty degeneration of the liver; JN, physiological structure of the liver and presence of multiple small foci of fatty degeneration, a single cluster of mononuclear cell infiltration; JNH, multiple small fatty degeneration of the liver, combined with multiple clusters of mononuclear cell infiltration; SN, presence of multiple small foci of fatty degeneration, a single cluster of mononuclear cell infiltration; SNH, single-focal fatty degeneration of the liver, combined with single clusters of mononuclear cell infiltration.

4. Discussion

of fatty degeneration; CNH, extensive multiple-multifocal foci of fatty degeneration of the liver; PN, extensive singular fatty degeneration of the liver; PNH, extensive multifocal fatty degeneration of the liver; JN, physiological structure of the liver and presence of multiple small foci of fatty degeneration, a single cluster of mononuclear cell infiltration; JNH, multiple small fatty degeneration of the liver, combined with multiple clusters of mononuclear cell infiltration; SN, presence of multiple small foci of fatty degeneration, a single cluster of mononuclear cell infiltration; SNH, single-focal fatty degeneration of the liver, combined with single clusters of mononuclear cell infiltration.

4. Discussion

The present experiment involves Wistar rats (Cmdb:Wi) because the rat model is an established host model for nutritional and metabolic studies, including the gastrointestinal response to nutritional interventions and the systemic (metabolic) changes observed with test dietary supplementation. Of course, there are limitations of applying findings from an animal study to humans. These limitations stem from differences in species, fiber, and copper content of the diet, and the inability to test dietary nanoparticles on humans. However, sufficient literature supports the statement that the rat model provides important strengths for the study of human health and disease in relation to human dietary habits and environment. Copper is essential for numerous metabolic activities, including lipid metabolism, redox balance, and iron mobilization, and its homeostasis is crucial for maintaining cellular function [23,24]. Dysregulation of copper levels can lead to oxidative stress, which is implicated in developing many liver metabolic disorders. Recently, there has been much interest in using nanocompounds in food. Due to their size and surface interaction area, these compounds may affect organisms differently than their native form. Indeed, in this study, replacing $CuCO_3$ with CuNPs at a dose of 6.5 mg/kg significantly reduced fat, cholesterol, and triglycerides in the liver, while increasing the dose to 13 mg/kg showed the opposite effect. Due to the enhanced interaction of nano Cu with the body, they have a more significant impact, which may be comparable to an excess of copper in the diet. Other studies also showed that excess Cu could induce mitochondrial dysfunction, promoting lipid deposition and lipogenesis [25]. Furthermore, histological analysis confirmed the observed changes regarding the effect of CuNPs dose on hepatic lipid metabolism. Numerous multifocal foci of lipid degeneration were noted in the group with an increased dose of CuNPs. Irrespective of the dose, CuNPs increased plasma aspartate aminotransferase activity, which may indicate the activation of inflammatory mechanisms in the liver. These changes were most likely related to the increased expression levels of *srebp-1c* and *cox-2* in groups with CuNPs, which are involved in lipid accumulation mechanisms and liver inflammation development [26,27]. Srebp-1c is intricately linked to insulin signaling and plays a pivotal role in lipid and glucose metabolism. Insulin stimulates the transcription of the *srebp-1c* gene, activating the transcription of the genes necessary for fatty acid synthesis, thereby promoting de novo lipogenesis in the liver [26,28]. Interestingly, in this study, blood insulin levels decreased significantly, while glucose levels did not change when the dietary source of Cu was replaced with CuNPs. Other studies showed that srebp-1c in the liver represses insulin receptor substrate-2 (IRS-2) transcription by displacing forkhead proteins from the insulin response element on the IRS-2 promoter, contributing to hepatic insulin resistance [29]. This indicates that an increased dose of CuNPs can significantly interfere with the regulation of glucose and lipid metabolism in the liver. This may disrupt the mechanism linking the interaction of *srebp-1c* and insulin, thus increasing liver fat accumulation and developing inflammation.

Our previous nutritional study with CuNPs suggests that adding dietary fiber to rats' diets decreases Cu intake, affecting Cu bioavailability [20]. Based on these results, it was assumed that combining CuNPs with dietary fibers may regulate the intensity of the nanocompound's action on hepatic metabolism. Indeed, this study confirmed that the beneficial effects of combining CuNPs with dietary fiber depend on the type of fiber. The combination of psyllium and CuNPs in the diet, regardless of dose, was most beneficial in reducing fat, total cholesterol, and triglycerides in the liver. In addition, a comparable

effect was observed when inulin was combined with an increased dose of CuNPs in the diet. While inulin and psyllium offer significant health benefits, their nutritional and functional properties differ. Inulin is primarily valued for its prebiotic effects and its role in enhancing mineral absorption, metabolic health, and reducing inflammation [30]. In contrast, psyllium is prized for its ability to form a gel-like substance in the gut that helps slow digestion, manage blood sugar levels, and lower cholesterol [31]. Therefore, adding fibers like psyllium and inulin to the diet could be an explanation for the mitigation of the CuNPs effect by reducing the activity of aminotransferases in the blood. Additionally, higher blood HDL levels and lower atherogenicity factor values were observed in the groups with CuNPs supplemented with psyllium and inulin. The same was observed in the histological analysis, where adding inulin and psyllium to a diet with a higher dose of CuNPs reduced the foci of fatty degeneration of the liver. The beneficial effects of combining psyllium with CuNPs on lipid metabolism and activating pro-inflammatory mechanisms in the liver are most likely related to the observed downregulation of *srebp1c*, *ppar-γ*, and *cox-2* expression levels. These molecule indicators are involved in many mechanisms regulating lipids, glucose metabolism, and inflammation in the liver [26,32]. Particularly interesting are the changes in *ppar-γ* expression. Activation of this receptor in the liver can lead to the increased storage of fatty acids in the form of triglycerides, which is a protective mechanism against lipotoxicity, preventing the accumulation of free fatty acids that can cause cellular damage [33,34]. This mechanism may explain why liver triglyceride levels were reduced in the group with psyllium.

Moreover, *ppar-γ* plays a crucial role in glucose metabolism. It enhances insulin sensitivity by modulating the expression of genes involved in glucose uptake and utilization. It is essential in developing NAFLD and type 2 diabetes, where insulin resistance is a standard disorder. By improving insulin sensitivity, *ppar-γ* activation can help reduce hepatic glucose production and improve overall glucose homeostasis [35,36]. Interestingly, when the examined fibers were added to the diets, CuPNs' insulin-lowering effect with unchanged blood glucose levels was also observed. However, the examined dietary fibers reduced glucagon levels. Glucagon is produced by the alpha cells of the pancreas and serves to increase blood glucose levels, acting as a counter-regulatory hormone to insulin [37]. Consequently, the natural mechanism for lowering blood insulin levels involves a decrease in glucagon secretion. This mechanism was disrupted when CuNPs were added to the diet. However, adding dietary fibers (specifically psyllium) reversed the action of CuNPs and lowered glucagon levels.

Another mechanism that can indirectly influence blood insulin levels is related to ghrelin secretion. Ghrelin has been shown to inhibit insulin secretion, which can lead to increased blood glucose levels and potentially contribute to insulin resistance over time [38]. When comparing the dietary fibers tested, psyllium showed the most significant reduction in blood ghrelin levels. However, inulin increases the level of this hormone. This effect may also explain the lowest insulin level observed in the group with CuNPs and inulin. One of the mechanisms regulating ghrelin secretion is associated with the feeling of satiety. Therefore, the characteristic gelling effect of psyllium [31], which can make it easier to feel full, may also reduce the secretion of this hormone. The effect of a decrease in ghrelin levels matches the reduced dietary intake in the psyllium and CuNPs group described in an earlier paper [17]. For inulin, the mechanism of increasing ghrelin levels in blood is probably associated with its prebiotic properties and the production of short-chain fatty acids (SCFAs). The fermentation of inulin by gut bacteria increases the production of SCFAs, which can enhance the secretion of ghrelin by stimulating the enteroendocrine cells in the stomach and the small intestine. Moreover, the increase in SCFAs can also affect the expression of genes involved in ghrelin production [39–41].

5. Conclusions

In conclusion, the gathered data show that CuNPs can significantly impact hepatic lipid metabolism and inflammatory mechanisms in the liver. The study revealed that

replacing $CuCO_3$ with CuNPs at different doses had distinct, unfavorable effects on liver fat, cholesterol, and triglyceride levels. High doses of CuNPs induced lipid degeneration and activated inflammatory mechanisms, as indicated by the increased plasma aspartate aminotransferase activity and the elevated expression levels of specific genes associated with lipid accumulation and liver inflammation. Interestingly, the study also demonstrated that combining CuNPs with dietary fibers, such as psyllium and inulin, modulates the effects of CuNPs on hepatic metabolism. Combining CuNPs with these dietary fibers showed beneficial effects in reducing fat, cholesterol, and triglycerides, while also decreasing the activity of aminotransferases in the blood. Moreover, it was observed that adding psyllium and inulin resulted in higher blood HDL levels and lower liver fatty degeneration foci when combined with CuNPs. These findings underscore the intricate relationship between CuNPs, dietary fibers, and hepatic metabolism. The addition of psyllium to the diet with CuNPs downregulated specific gene expression levels, such as *srebp-1c*, *ppar-γ*, and *cox-2*, responsible for the regulation of lipids, glucose metabolism, and inflammatory pathways in the liver. The study highlights the potential of dietary fibers in reducing the negative effects of CuNPs on liver function, opening the door for further research into the combined effects of nanocompounds and dietary interventions on metabolic health. However, it is important to note that the potential toxicity of CuNPs still poses a significant barrier to clinical translation.

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Institutional Review Board Statement: The animal study protocol was approved by the National Ethics Committee for Animal Experiments (Permission No. 19/2021; Olsztyn, Poland, 17 March 2021). The study was conducted in accordance with the ARRIVE guidelines, and every attempt was made to minimize the animals' suffering during the experiment.

Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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antioxidants

Article

Inulin Improves the Redox Response in Rats Fed a Diet Containing Recommended Copper Nanoparticle (CuNPs) Levels, While Pectin or Psyllium in Rats Receive Excessive CuNPs Levels in the Diet

Aleksandra Marzec [1], Ewelina Cholewińska [1,*], Bartosz Fotschki [2], Jerzy Juśkiewicz [2] and Katarzyna Ognik [1,*]

[1] Department of Biochemistry and Toxicology, Faculty of Animal Sciences and Bioeconomy, University of Life Sciences in Lublin, Akademicka 13, 20-950 Lublin, Poland; aleksandra.marzec@up.lublin.pl
[2] Division of Food Science, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Trylińskiego 18, 10-683 Olsztyn, Poland; b.fotschki@pan.olsztyn.pl (B.F.); j.juskiewicz@pan.olsztyn.pl (J.J.)
* Correspondence: ewelina.cholewinska@up.lublin.pl (E.C.); katarzyna.ognik@up.lublin.pl (K.O.)

Abstract: The study aimed to determine the effect of dietary inclusion of the recommended (6.5 mg Cu/kg diet) or double the recommended (13.0 mg Cu/kg diet) levels of copper nanoparticles (CuNPs) in combination with different types of dietary fibre on selected redox status indicators in the blood and tissues of male Wistar rats. Control groups were fed diets containing cellulose and a mineral mixture with standard or enhanced content of $CuCO_3$. The experimental groups were fed a diet supplemented with CuNPs (6.5 or 13 mg/kg) and combined with various fibre types—cellulose, pectin, inulin, or psyllium. After the feeding period, rats' organs were collected to assess selected indicators of redox status. The obtained results suggest that the addition of dietary fibre in the form of inulin may beneficially stimulate the response of the redox system in the conditions of CuNPs nutrition at the recommended dose, pectin, or psyllium in the case of an excessive supply of CuNPs in the diet. Thus, selecting the appropriate type of dietary fibre based on the CuNPs' level in the diet may effectively protect the organism from the potentially harmful prooxidative effect of CuNPs, ultimately contributing to a favourable regulation of their metabolic impact in the body.

Keywords: copper nanoparticles; inulin; pectin; psyllium; cellulose; redox status; blood; tissues

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1. Introduction

Nanotechnology, a rapidly advancing scientific field, is finding increasingly broad applications across various sectors, including medicine, the food industry, electronics, and environmental protection [1]. Nanoparticles have also garnered growing interest among nutritionists due to their potential dietary use, which offers new avenues for enhancing health, primarily by improving nutrient bioavailability [2]. Copper (Cu) is an example of a micronutrient for which dietary supplementation is necessary to ensure the proper functioning of the body. However, the conventional, standardly used Cu inorganic forms have relatively poor absorption in the organism [3,4]. The existing animal studies indicate that incorporating copper nanoparticles (CuNPs) into the diet may significantly enhance the absorption and utilisation of copper within the body [3–6]. However, dietary inclusion of copper nanoparticles in animals may pose certain risks. The current literature suggests that CuNPs may exhibit some toxicity towards living organisms [5,7,8], which is likely

attributable, to a significant extent, to their capacity for inducing oxidative stress [9]. It is hypothesised that in the gastrointestinal tract lumen, CuNPs may dissociate into ionic forms, which, by catalysing the Fenton reaction, lead to the generation of highly reactive oxygen species [10–12]. The resulting increasing oxidative stress may lead to the loss of cellular functionality through lipid peroxidation, metabolic enzyme dysfunction, damage to protein structures, and DNA mutations [13–15]. Our previous research confirmed that replacing conventional $CuCO_3$ with CuNPs in rat diets can enhance lipid peroxidation processes and impair antioxidant defences in the lungs and kidneys [16]. Nonetheless, this replacement contributed to reduced lipid peroxidation in the brain and liver and decreased protein oxidation and nitration, as well as DNA oxidation and methylation in the blood of the studied animals [17]. Given that the inclusion of copper nanoparticles in animal diets can exert both positive and negative impacts on certain aspects of the organism's redox status, it appears plausible that this effect could be additionally beneficially modulated through dietary fibre incorporation. This common dietary component has demonstrated antioxidant properties [18–21]. The available literature suggests that prebiotic fibres, such as inulin—and to a lesser extent, pectin and psyllium—promote the production of short-chain fatty acids (SCFAs) during fermentation in the colon, which may exert antioxidant effects [19,20]. Additionally, inulin supports the growth of beneficial gut bacteria that produce vitamins essential for properly functioning antioxidant enzymes [20]. Dietary fibre may also bind heavy metals and other ions, including Cu ions in the small intestine, which could positively regulate CuNPs' bioavailability while reducing the free radicals generated within the organism [21–24]. The results of our previous studies align with these findings, demonstrating that dietary fibre supplementation strengthens the intestinal barrier in rats receiving CuNPs in their diet [25]. Furthermore, adding dietary fibres such as pectin, inulin, or psyllium to a diet containing CuNPs at concentrations twice the nutritional recommendation helps maintain copper homeostasis within the organism by significantly modifying the absorption and excretion of this trace element [6].

It was hypothesised that a dietary combination of CuNPs with various types of fibre—neutral cellulose (control), prebiotic inulin, viscous pectin, or swelling psyllium—would influence the redox response, thereby modulating the metabolic effects of CuNPs within the organism. The study aimed to test this hypothesis by evaluating the impact of dietary inclusion of the recommended (6.5 mg Cu/kg diet) or double the recommended (13.0 mg Cu/kg diet) levels of CuNPs in combination with different types of dietary fibre—cellulose, pectin, inulin, or psyllium—on selected redox status indicators in the blood and tissues of rats. In terms of animal models, there is a notable similarity between the internal organs of rodents and humans, particularly regarding redox status. While in vivo data on metal nanoparticles derived from rats may not be directly applicable to humans, it is important to emphasise that this new information enhances our understanding in the field.

2. Materials and Methods

The study presented in this paper is part of a large research project aimed at determining the effects of the dietary combination of CuNPs with various fibre types—cellulose, inulin, pectin, or psyllium on multiple aspects of the biological response in rats. Therefore, the experiment's design and the procedures used have previously been published in scientific articles [6,26–28].

2.1. Materials' Characterisation: Copper Nanoparticles and Fibre Types

Commercially available copper nanoparticles (CuNPs) supplied by SkySpring Nanomaterials, Inc. (Houston, TX, USA) were used in the study. These CuNPs were selected because of their well-documented stability and clearly defined physicochemical properties,

including a purity of 99.9% trace metal basis, the appearance of addle brown nanopowder, an aerodynamic Particle Size (APS) of 40–60 nm, a specific surface area (SSA) of ~12 m^2/g, a spherical morphology, bulk density of 0.19 g/cm^3, and true density: 8.9 g/cm^3, without the presence of additional coatings, surfactants, or surface modifiers. The experiment used the same CuNPs as those used in our previous studies [4,6,16,17,26–28]. $CuCO_3$, serving as the control source of dietary copper, was obtained from Merck KGaA (Darmstadt, Germany). α-Cellulose (Sigma, Poznań, Poland) was used as the control dietary fibre. The experimental diets included the following fibre sources: pectin (PectinE 440(I), Brouwland, Beverlo, Belgium), inulin (Frutafit Tex, Sensus, 's-Hertogenbosch, The Netherlands), and psyllium (Psyllium husk powder, NaturaleBio, Rome, Italy).

2.2. Animal Study Protocol and Diet Composition

All procedures involving animals were carried out following Polish regulations on animal experimentation and ethical standards, and were fully compliant with Directive 2010/63/EU of the European Convention for the Protection of Vertebrate Animals for Experimental and Other Scientific Purposes [29]. The Local Ethics Committee for Animal Experiments in Olsztyn approved the experimental protocol (Approval No. 19/2021; Olsztyn, Poland; Approval date: 17 March 2021). The protocol containing the research questions, experimental schema with the in vivo study design, and analysis plan was submitted to the reviewers of the National Science Center (Kraków, Poland) for evaluation, and was subsequently approved and funded.

The study involved one hundred healthy outbred male Wistar rats Cmdb:Wi, obtained from a certified breeding facility (breeder register 051, Institute of Animal Reproduction and Food Research PAS, Olsztyn, Poland). After a two-week acclimatisation period, they were randomly divided into ten groups, each consisting of ten rats. The environment included a 12 h light–dark cycle, a temperature maintained at 22 ± 1 °C, a relative humidity ranging from 45% to 65%, and 15 air changes per hour. Random numbers were created using Microsoft Excel's standard = RAND() function. In the room with the animals, the cages were placed so that the same number of rats from a given group was placed in respective places on the cage rack (top, bottom, left side, right side). The rats 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 (see Table 1 for details). In the control diets, $CuCO_3$ was incorporated into the mineral mix at a standard and high level (6.5 and 13 mg/kg diet). In CuNP-supplemented groups, $CuCO_3$ was excluded from mineral mixtures, and CuNPs were added to diets as an emulsion in rapeseed oil to ensure safe handling. The control dietary fibre was α-cellulose at 8% of the diet. Experimental fibres—pectin (viscous), inulin (prebiotic), and psyllium (bulking)—were included at 6%, replacing a portion of the cellulose. The experimental protocol consisted of 10 groups, n = 10 per group. The sample size was determined based on our own previous research. A single animal was considered an experimental unit.

The study was conducted following the ARRIVE guidelines [30], and all possible measures were taken to minimise the suffering of the animals used in the experiment. During the period of experimental feeding, in the event of adverse effects related to humane endpoints, i.e., cessation of diet intake for more than 2 days, making specific sounds as a pain signal for more than 1 h, the appearance of neurological symptoms (e.g., ataxia, impairment in maintaining a favorable body position), and the presence of blood in the feces for more than 1 day, a veterinarian (employed for these purposes at the institute) may make a decision of humane euthanasia using the method of gradual filling of the chamber

with the animal with carbon dioxide or the method of dislodging the cervical vertebrae of a previously sedated animal. To the best of our knowledge, based on the literature and our previous experiments, the above symptoms should not be related to the experimental factors (fiber, nanoparticles), and there is only a minimal risk of their occurrence.

After 6 weeks of dietary treatments, rats were fasted for 8 h and then anaesthetised *i.p.* with ketamine and xylazine (K, 100/kg BW; X, 10 mg/kg BW). None of the animals were excluded from the experiment. It was assumed that for each experimental group, the criterion for removing an animal from the experiment was humane endpoints. The project manager was the only person who was aware of the animal's allocation to a particular study group. Not all of the analysis contractors were acquainted with the treatment animal allocation. Blood was drawn from the caudal vena cava into both EDTA and heparinised tubes. Animals were subsequently sacrificed by cervical dislocation, and major organs such as the heart, lungs, jejunum, liver, pancreas, kidneys, spleen, and testes were collected. Blood plasma was obtained by chilling and centrifugation at 350× *g* for 10 min at 4 °C. Tissue homogenates were prepared by homogenising 1 g of each organ sample in 9 mL of phosphate-buffered saline (PBS), followed by centrifugation (3000× *g*, 10 min, 4 °C). Supernatants obtained from centrifuged homogenates and plasma samples were stored at −80 °C until further analysis.

Table 1. The composition of experimental diets administered to rats for 6 weeks (this table was also published in [6,26–28].

	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

[1] Casein preparation: crude protein 89.7%, crude fat 0.3%, ash 2.0%, and water 8.0%. [2] α-Cellulose (SIGMA, Poznan, Poland), the main source of dietary fibre. [3] AIN-93G-VM [31], g/kg mix: 3.0 nicotinic acid, 1.6 Ca pantothenate, 0.7 pyridoxine-HCl, 0.6 thiamin-HCl, 0.6 riboflavin, 0.2 folic acid, 0.02 biotin, 2.5 vitamin B-12 (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 D-3 (cholecalciferol, 400,000 IU g^{-1}), 0.075 vitamin K-1 (phylloquinone), 974.655 powdered sucrose. [4] In the experimental treatments with CuNPs, the MX was deprived of $CuCO_3$ to keep the operator safe while preparing the experimental diets, and the CuNP preparation was added as an emulsion along with dietary rapeseed oil. Such a procedure was successfully applied in the previous experiments. [5] Maize starch preparation: crude protein 0.6%, crude fat 0.9%, ash 0.2%, total dietary fibre 0%, and water 8.8%.

2.3. Blood and Tissue Analyses

In blood plasma, levels of selected redox status indicators including superoxide dismutase (SOD), catalase (CAT), ceruloplasmin (Cp), total antioxidant status (TAS), malondialdehyde (MDA), 3-nitrotyrosine (3-NT), protein carbonyl derivatives (PCs), 8-hydroxydeoxyguanosine (8-OHdG), DNA repair enzymes—Apurinic/Apyrimidinic Endonuclease 1 (APE-1) and 8-oxoguanine DNA glycosylase (OGG1)—and markers of apoptotic cell death, caspase 3 (Casp3) and caspase 8 (Casp8), were measured. The measurements were performed using a commercially available enzyme-linked immunosorbent assays (ELISA) kit, following the

manufacturer's instructions (Shanghai Qayee Biotechnology Co., Ltd., Shanghai, China). Absorbance readings were taken at 450 nm using an ELISA microplate reader (Sunrise™, Tecan Group Ltd., Männedorf, Switzerland). For global DNA methylation analysis, DNA was first isolated from blood using the Blood Mini Kit column-based extraction system (A&A Biotechnology, Gdańsk, Poland), according to the manufacturer's protocol. Then, global DNA methylation levels were subsequently assessed in the isolated DNA using commercial diagnostic kits provided by Sigma-Aldrich (Taufkirchen, Germany). The activities of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT), along with the concentration of malondialdehyde (MDA) as an indicator of lipid peroxidation, were assessed in homogenates of the heart, lungs, jejunum, liver, pancreas, kidneys, spleen, and testes, following the procedure outlined by Ognik and Wertelecki [32].

2.4. Data Analysis and Statistics

Statistical analyses were performed using STATISTICA software, version 12.0 (StatSoft Corp., Krakow, Poland). A two-way ANOVA was conducted to evaluate the effects of two main factors: the CuNPs dose (L: 6.5 mg/kg and H: 13 mg/kg) and the type of dietary fibre (cellulose, pectin, inulin, and psyllium), followed by Duncan's multiple range post hoc test. In addition, *t*-tests were applied to compare each group receiving the lower CuNP dose (L) with the control group C (fed 6.5 mg/kg Cu from $CuCO_3$ and cellulose as the fibre source), and to compare groups receiving the higher CuNPs dose (H) with the control group CH (fed 13 mg/kg Cu from $CuCO_3$ with cellulose). Differences were considered statistically significant at $p \leq 0.05$. SEM, the pooled standard error of the mean, was calculated as the standard deviation for all rats divided by the square root of rat number, $n = 100$.

3. Results

3.1. One-Way ANOVA

3.1.1. C vs. CN, PN, JN i SN

The one-way analysis comparing the experimental groups CN, PN, JN, and SN to the control group C revealed a significant reduction in SOD levels in the blood plasma of all experimental groups (CN, PN, JN, and SN) compared to the control. Simultaneously, CAT levels were increased in the plasma of the CN, PN, and JN groups. In the plasma of rats in the PN and SN groups, a decrease in Cp levels alongside an increase in MDA levels compared to the control group C was noted. Additionally, in the blood plasma of rats from the PN, JN, and SN groups, higher levels of 8-OHdG were found than in the control group (C). Lower plasma PC levels were observed in the PN group compared to the C group. In turn, DNA methylation levels in the blood were decreased in the CN and SN groups vs. the C group. Lower levels of APE-1 and OGG-1 were observed in the blood plasma of rats from the SN group than in the control group C. A decreased level of APE-1 in the blood plasma was also observed in rats from the CN and JN groups. In the SN group, a decrease in the level of CASP3 and CASP8 in the blood was observed, whereas in the blood of rats from the JN group, only the level of CASP3 decreased compared to the C group (Table 2). In the CN group, an increase in MDA levels was recorded in the testes, while their levels in intestinal tissue and liver decreased compared to the control group (C). Relative to the control group (C), the MDA levels in the PN group increased in the heart, lungs, kidneys, spleen, and testes, but decreased in the liver. The JN group showed reduced MDA levels in the heart, lungs, intestinal tissue, liver, pancreas, and spleen vs. the C group. In contrast, the SN group exhibited increased MDA levels in the liver, spleen, and testes, with a concurrent decrease in the intestinal tissue compared to the control group (C) (Figure 1; see Supplementary Materials—Table S1 for details). In the CN group, an increase in SOD activity was noted in the kidneys; meanwhile, the decreased activity of

this enzyme was detected in the heart, lungs, intestinal tissue, and liver compared to the C group. Relative to the control group (C), SOD activity in the PN group increased in the pancreas and kidneys but decreased in the lungs. The JN group showed increased SOD activity in the lungs, pancreas, and kidneys, alongside a decrease in this enzyme activity in the heart and spleen vs. the C group. In the SN group, increased SOD activity was observed in the pancreas and kidneys, while it decreased in the heart and liver compared to the control group (C) (Figure 2; see Supplementary Materials—Table S2 for details). In the CN group, CAT activity increased in the heart, intestinal tissue, liver, and pancreas but decreased in the lungs, kidneys, and spleen relative to the control group (C). Relative to control group C, CAT activity in the PN group increased in the liver but decreased in the heart, intestinal tissue, kidneys, and spleen. The JN group exhibited increased CAT activity in the intestinal tissue and liver, with lowered activity in the kidneys and testes vs. the C group. In the rats from the SN group, increased CAT activity was noted in the liver; meanwhile, its activity decreased in the intestinal tissue, kidneys, and testes compared to the control group (C) (Figure 3; see Supplementary Materials—Table S3 for details).

Table 2. Blood parameters in rats fed experimental diets (n = 10 per group) *.

	SOD	CAT	Cp	TAS	MDA	3-NT	PC	8-OHdG	DNA Methylation	APE-1	OGG1	Casp3	Casp8
	ng/mL	ng/mL	U/L	mmol/L	nmol/mL	ng/mL	nmol/mg Protein	ng/mL	%	ng/mL	pg/mL	ng/mL	ng/mL
Control C	36.6	27.1	164	0.902	1.49	14.8	4.75	6.93	1.46	180	493	434	102
Control CH	17.2	29.7	125	0.973	1.5 6	14.7	3.36	7.80	1.16	171	460	441	100
2-way ANOVA:													
CN	17.3 $^{\#}$	31.7 $^{\#}$	141	0.932	1.41 ^{d}	14.6	4.82	7.93	0.853 $^{b\#}$	167 $^{a\#}$	454	402 ^{a}	99.3
CNH	18.6	31.4	148 $^{\&}$	0.974	1.50 ^{cd}	14.6	4.20	7.76	1.52 ^{a}	167 ^{a}	460	387 $^{ab\&}$	97.7
PN	8.31 $^{\#}$	33.5 $^{\#}$	131 $^{\#}$	1.08	1.83 $^{bc\#}$	15.8	3.35 $^{\#}$	8.07 $^{\#}$	1.28 ^{a}	169 ^{a}	471	411 ^{a}	123
PNH	10.7 $^{\&}$	32.2	125	0.943	1.64 ^{cd}	18.4 $^{\&}$	3.03	8.93 $^{\&}$	0.915 ^{b}	155 ^{ab}	386 $^{\&}$	335 $^{c\&}$	101
JN	5.04 $^{\#}$	31.9 $^{\#}$	162	0.956	1.63 ^{cd}	14.8	4.35	7.90 $^{\#}$	1.59 ^{a}	153 $^{ab\#}$	428	347 $^{bc\#}$	105
JNH	8.18 $^{\&}$	29.9	158 $^{\&}$	0.968	2.87 $^{a\&}$	15.2	4.75 $^{\&}$	8.45	0.909 ^{b}	152 $^{ab\&}$	408	350 $^{bc\&}$	93.3
SN	6.99 $^{\#}$	31.1	138 $^{\#}$	0.954	2.07 $^{b\#}$	16.8	5.29	9.05 $^{\#}$	0.966 $^{b\#}$	143 $^{b\#}$	398 $^{\#}$	330 $^{c\#}$	77.9 $^{\#}$
SNH	6.56 $^{\&}$	31.0	129	1.09	1.36 $^{d\&}$	17.2	5.01 $^{\&}$	8.62	0.845 ^{b}	162 ^{a}	399 $^{\&}$	350 $^{bc\&}$	83.7
SEM	0.986	0.389	2.083	0.019	0.054	0.323	0.139	0.119	0.044	1.934	7.941	6.108	2.934
CuNPs dose (D)													
L (6.5 mg/kg)	9.42	32.1	143	0.981	1.74	15.5	4.45	8.24	1.17	158	438	373	101
H (13 mg/kg)	11.0	31.1	140	0.995	1.84	16.4	4.25	8.44	1.05	159	413	356	93.8
p value	0.065	0.242	0.239	0.724	0.197	0.268	0.480	0.406	0.110	0.737	0.137	0.132	0.309
Fibre type (F)													
C (cellulose)	18.0 ^{a}	31.6	144 ^{b}	0.953	1.45	14.6 ^{b}	4.52 ^{a}	7.84 ^{b}	1.19	167	457	394	97.3 ^{ab}
P (pectin)	9.53 ^{b}	32.9	128 ^{c}	1.01	1.74	17.1 ^{a}	3.19 ^{b}	8.50 ^{ab}	1.10	162	428	373	112 ^{a}
J (inulin)	6.61 ^{c}	30.9	160 ^{a}	0.962	2.25	15.0 ^{ab}	4.55 ^{a}	8.17 ^{ab}	1.25	153	418	349	98.9 ^{ab}
S (psyllium)	6.77 ^{c}	31.0	133 ^{c}	1.02	1.71	17.0 ^{a}	5.15 ^{a}	8.84 ^{a}	0.906	153	399	340	80.8 ^{b}
p value	<0.001	0.309	<0.001	0.513	<0.001	0.033	<0.001	0.028	0.015	0.024	0.090	0.004	0.017
Interaction D × F													
p value	0.488	0.837	0.196	0.117	<0.001	0.607	0.649	0.192	<0.001	0.037	0.192	0.019	0.445

* 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 $CuCO_3$, respectively) with 8% of cellulose as dietary fibre source; groups CN and CNH, fed diets with supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively), with 8% of cellulose dietary fibre source; groups PN and PNH, fed diets with supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively), with 2% of cellulose and 6% of pectin dietary fibre source; groups JN and JNH, fed diets with supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively), with 2% of cellulose and 6% of inulin dietary fibre source; groups SN and SNH, fed diets with supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively), with 2% of cellulose and 6% of psyllium dietary fibre source; L, treatment (n = 40) with dietary CuNPs (6.5 mg/kg dose); H, treatment (n = 40) with dietary CuNPs (13 mg/kg dose); C, treatment (n = 20) with cellulose as dietary fibre; P, treatment (n = 20) with pectin as dietary fibre; J, treatment (n = 20) with inulin as dietary fibre; S, treatment (n = 20) with psyllium as dietary fibre; $^{a–d}$ Mean values within a column with unlike superscript letters are shown to be significantly different ($p < 0.05$); differences among the 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 6.5 mg/kg (CN, PN, JN, SN) was compared with the control C one with the aid of t-test ($^{\#}$ indicates a significant difference versus the C group); similarly, each experimental group fed CuNPs 13 mg/kg (CNH, PNH, JNH, SNH) was compared with the control CH one with the aid of t-test ($^{\&}$ indicates a significant difference versus the CH group); SEM, pooled standard error of mean (standard deviation for all rats divided by the square root of rat number, n = 100). SOD, superoxide dismutase; CAT, catalase; Cp, ceruroplasmin; TAS, total antioxidant capacity; MDA, malondialdehyde; 3-NT, 3-nitrotyrosine; PC, protein carbonyl derivatives; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; APE-1, apurinic/apyrimidinic endonuclease 1; OGG1, 8-oxoguanine glycosylase; Casp3, caspase 3; Casp8, caspase 8.

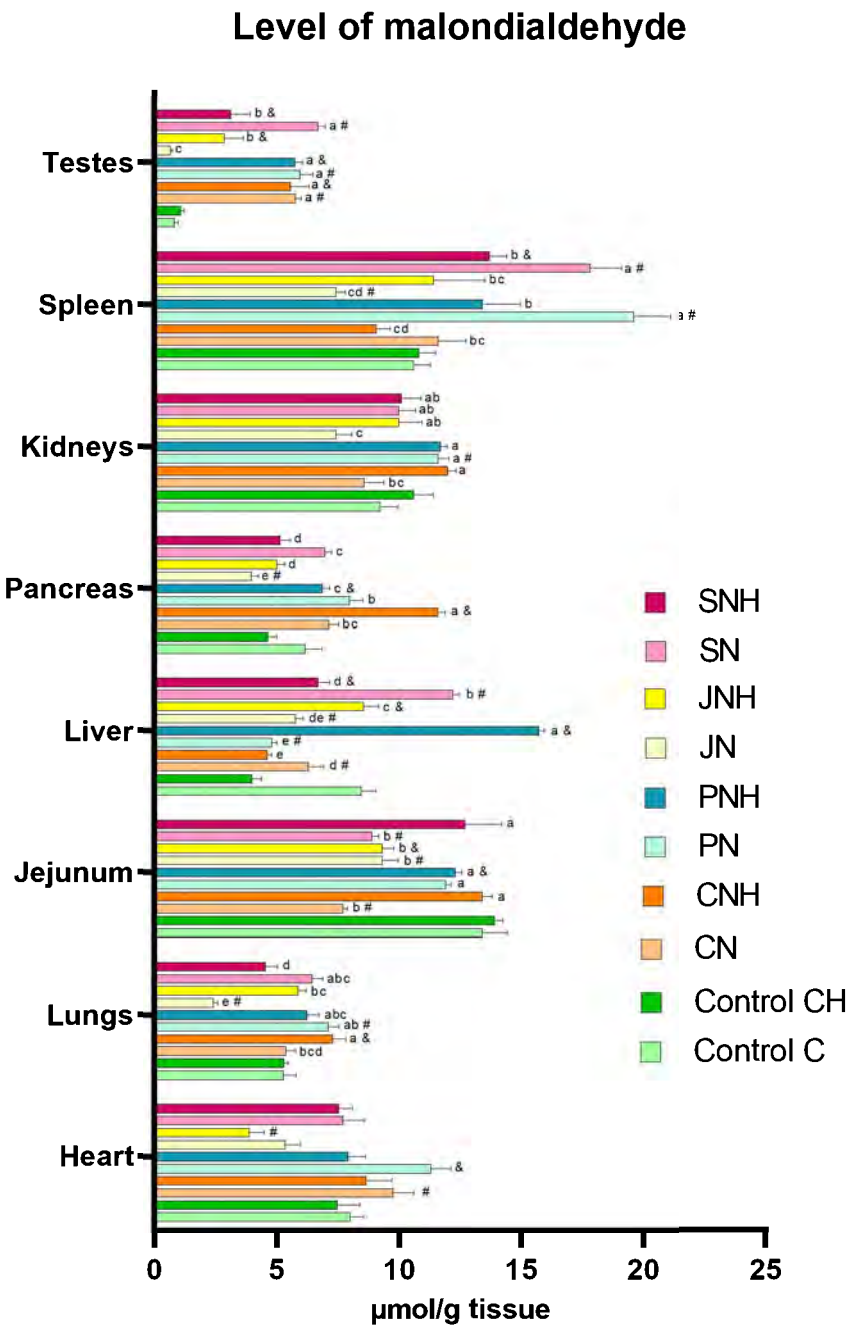

Figure 1. Level of malondialdehyde (MDA; µmol/g) in selected tissues in rats fed experimental diets (groups C and CH: control diets with standard (6.5 mg/kg) and enhanced (13 mg/kg) copper from $CuCO_3$, with 8% cellulose as fibre; groups CN and CNH: diets supplemented with CuNPs (6.5 and 13 mg/kg) plus 8% cellulose; groups PN and PNH: diets supplemented with CuNPs (6.5 and 13 mg/kg), with fibre from 2% cellulose and 6% pectin; groups JN and JNH: diets supplemented with CuNPs (6.5 and 13 mg/kg), with fibre from 2% cellulose and 6% inulin; groups SN and SNH: diets supplemented with CuNPs (6.5 and 13 mg/kg), with fibre from 2% cellulose and 6% psyllium). $^{a–e}$ Mean values within a column with unlike superscript letters are shown to be significantly different ($p < 0.05$); differences among the 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 6.5 mg/kg (CN, PN, JN, SN) was compared with the control C one with the aid of *t*-test ($^{\#}$ indicates a significant difference versus the C group); similarly, each experimental group fed CuNPs 13 mg/kg (CNH, PNH, JNH, SNH) was compared with the control CH one with the aid of *t*-test ($^{\&}$ indicates a significant difference versus the CH group).

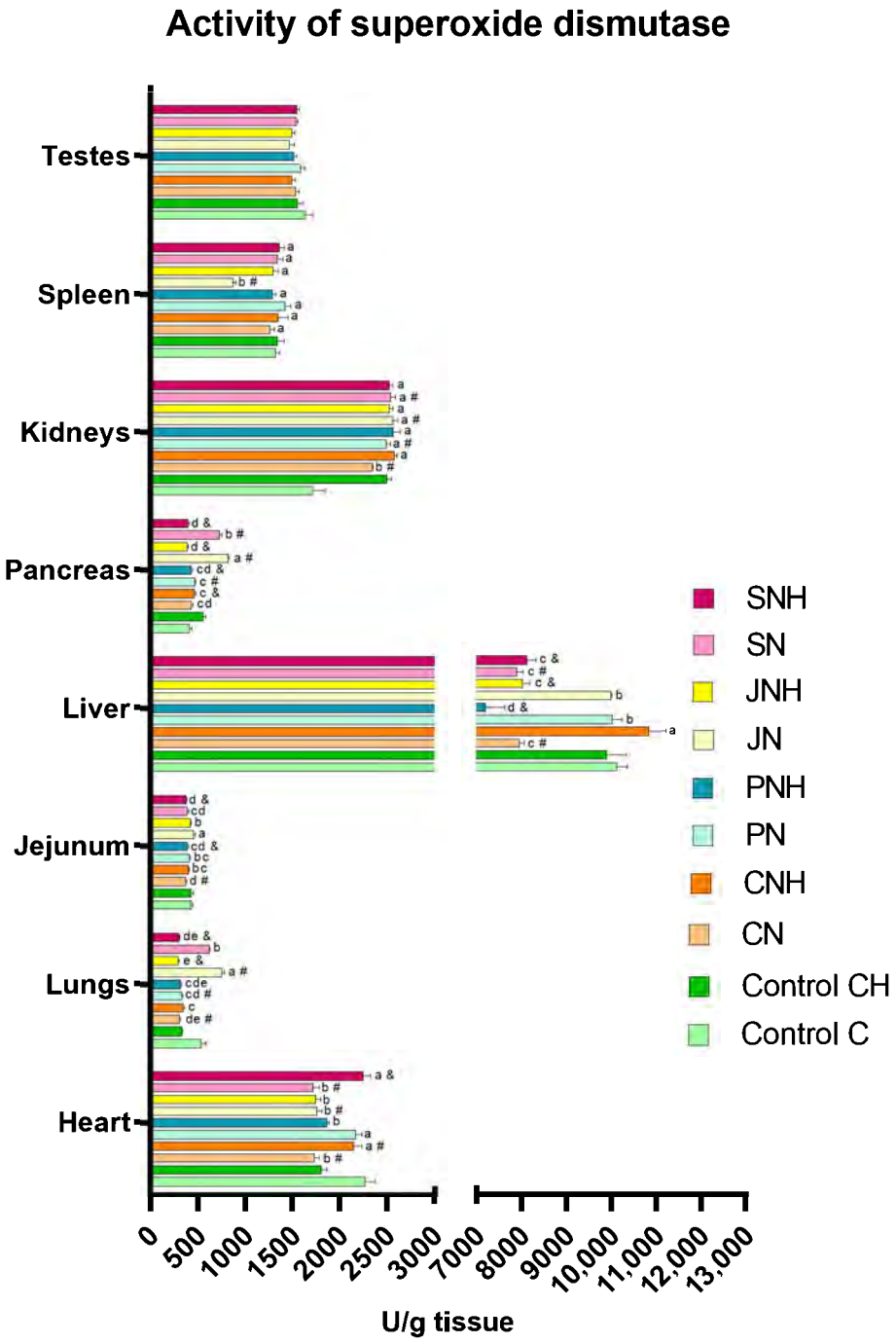

Figure 2. Activity of superoxide dismutase (SOD; U/g) in selected tissues in rats fed experimental diets (groups C and CH: control diets with standard (6.5 mg/kg) and enhanced (13 mg/kg) copper from $CuCO_3$, with 8% cellulose as fibre; groups CN and CNH: diets supplemented with CuNPs (6.5 and 13 mg/kg) plus 8% cellulose; groups PN and PNH: diets supplemented with CuNPs (6.5 and 13 mg/kg), with fibre from 2% cellulose and 6% pectin; groups JN and JNH: diets supplemented with CuNPs (6.5 and 13 mg/kg), with fibre from 2% cellulose and 6% inulin; groups SN and SNH: diets supplemented with CuNPs (6.5 and 13 mg/kg), with fibre from 2% cellulose and 6% psyllium). $^{a–e}$ Mean values within a column with unlike superscript letters are shown to be significantly different ($p < 0.05$); differences among the 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 6.5 mg/kg (CN, PN, JN, SN) was compared with the control C one with the aid of *t*-test ($^{\#}$ indicates a significant difference versus the C group); similarly, each experimental group fed CuNPs 13 mg/kg (CNH, PNH, JNH, SNH) was compared with the control CH one with the aid of *t*-test ($^{\&}$ indicates a significant difference versus the CH group).

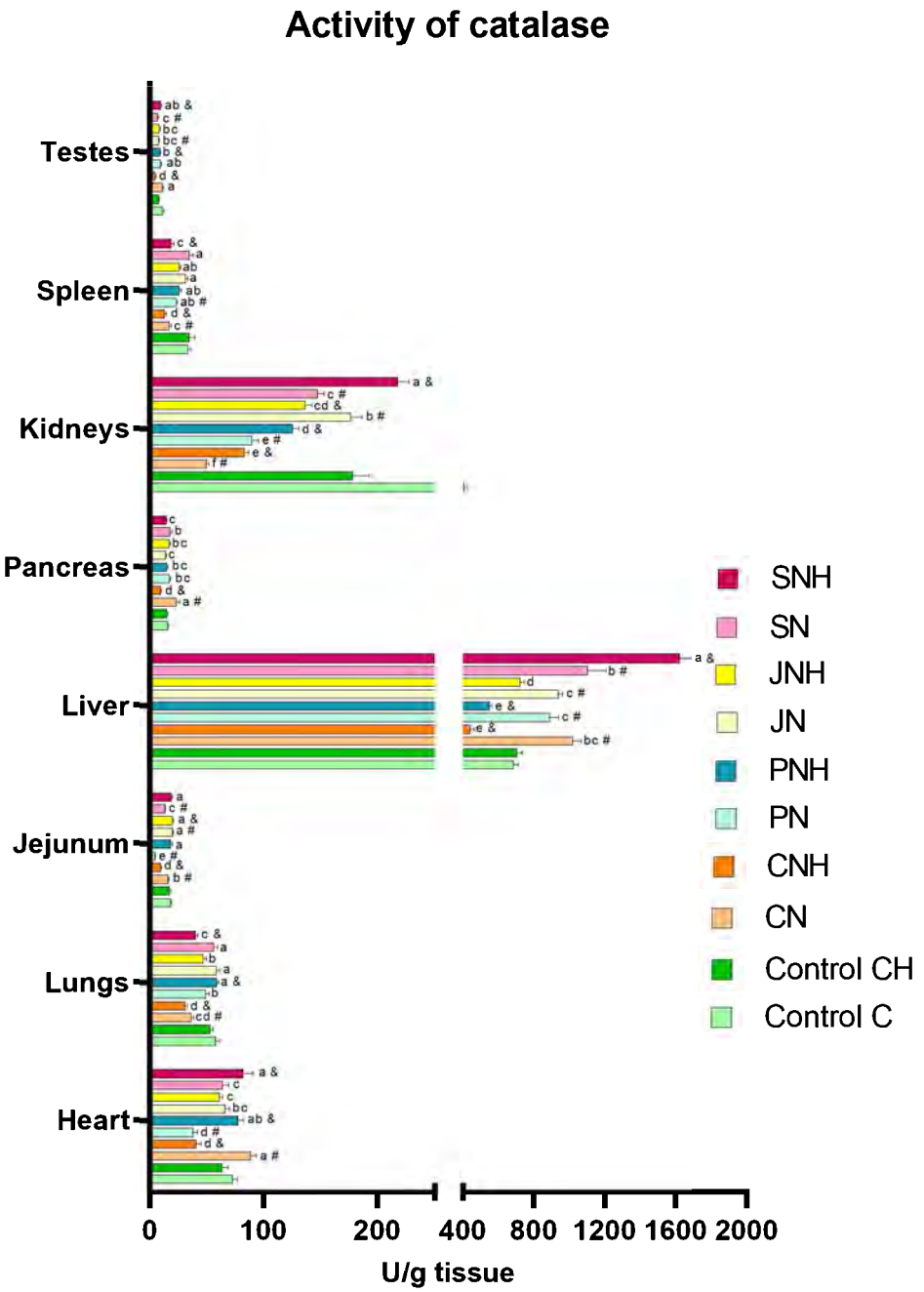

Figure 3. Activity of catalase (CAT; U/g) in selected tissues in rats fed experimental diets (groups C and CH: control diets with standard (6.5 mg/kg) and enhanced (13 mg/kg) copper from $CuCO_3$, with 8% cellulose as fibre; groups CN and CNH: diets supplemented with CuNPs (6.5 and 13 mg/kg) plus 8% cellulose; groups PN and PNH: diets supplemented with CuNPs (6.5 and 13 mg/kg), with fibre from 2% cellulose and 6% pectin; groups JN and JNH: diets supplemented with CuNPs (6.5 and 13 mg/kg), with fibre from 2% cellulose and 6% inulin; groups SN and SNH: diets supplemented with CuNPs (6.5 and 13 mg/kg), with fibre from 2% cellulose and 6% psyllium). $^{a–e}$ Mean values within a column with unlike superscript letters are shown to be significantly different ($p < 0.05$); differences among the 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 6.5 mg/kg (CN, PN, JN, SN) was compared with the control C one with the aid of *t*-test ($^{\#}$ indicates a significant difference versus the C group); similarly, each experimental group fed CuNPs 13 mg/kg (CNH, PNH, JNH, SNH) was compared with the control CH one with the aid of *t*-test ($^{\&}$ indicates a significant difference versus the CH group).

3.1.2. CH vs. CNH, PNH, JNH i SNH

The one-way analysis comparing experimental groups CNH, PNH, JNH, and SNH to the control group CH indicated a significant reduction in SOD levels in the blood plasma of rats from the PNH, JNH, and SNH groups. Additionally, an increase in Cp levels in the blood plasma of the CNH and JNH groups was observed. Relative to the CH group, the levels of 3-NT and 8-OHdG in the blood plasma of rats from the PNH group were elevated. The MDA levels in the blood plasma increased in the JNH group but decreased in the SNH group compared to the control group (CH). Furthermore, blood samples collected from rats from the JNH and SNH groups exhibited higher PC levels than the CH group. There was also a reduction in the APE-1 level in the JNH group vs. the CH group, the OGG-1 level in the PNH and SNH groups vs. the CH group, and the CASP3 level in all experimental groups (CNH, PNH, JNH, and SNH) vs. the CH group (Table 2). MDA levels were higher in rats' lungs, pancreas, and testes from the CNH group than in the rats from the control group (CH). Compared to the CH group, the MDA levels in the PNH group increased in the liver, pancreas, and testes but decreased in intestinal tissue. In the JNH group, rats exhibited increased MDA levels in the liver and testes while showing reduced levels in the heart and intestinal tissue relative to the control group (CH). In contrast, the SNH group demonstrated elevated MDA levels in the liver, spleen, and testes compared to the CH group (Figure 1; see Supplementary Materials—Table S1 for details). In the CNH group, increased SOD activity was noted in the heart; meanwhile, its activity was reduced in the pancreas compared to the control group (CH). Compared to the CH group, SOD activity in the PNH group decreased in the intestinal tissue, liver, and pancreas. Rats from the JNH group exhibited reduced SOD activity in the lungs, liver, and pancreas relative to the control group (CH). In turn, in the SNH group, SOD activity was increased in the heart but decreased in the lungs, intestinal tissue, liver, and pancreas (Figure 2; see Supplementary Materials—Table S2 for details). In the CNH group, CAT activity was reduced in the heart, lungs, intestinal tissue, liver, pancreas, kidneys, spleen, and testes relative to the control group (CH). Compared to the CH group, CAT activity in the PNH group increased in the heart, lungs, and testes, but its activity decreased in the liver and kidneys. In the JNH group, rats showed increased CAT activity in the intestinal tissue with reduced activity in the kidney compared to the CH group. The SNH group exhibited increased CAT activity in the heart, liver, and testes, while it decreased in the lungs, kidneys, and spleen compared to the CH group (Figure 3; see Supplementary Materials—Table S3 for details).

3.2. Two-Way ANOVA

The two-way ANOVA revealed the occurrence of significant interactions for MDA levels ($p < 0.001$), DNA methylation ($p < 0.001$), APE-1 ($p = 0.037$), and CASP3 ($p = 0.019$) in the blood (Table 2). Numerous interactions were also identified for MDA levels, as well as for SOD and CAT activities in various tissues (Figures 1–3; see Supplementary Materials—Tables S1–S3 for details). The occurrence of interactions were noted for MDA levels in the lung ($p < 0.001$), intestinal tissue ($p < 0.001$), liver ($p < 0.001$), pancreas ($p < 0.001$), kidney ($p = 0.020$), spleen ($p = 0.001$), and testis ($p < 0.001$) (Figure 1; see Supplementary Materials—Table S1 for details), and for SOD and CAT activity in the heart ($p < 0.001$; both), lung ($p < 0.001$; both), intestinal tissue ($p < 0.001$; both), liver ($p < 0.001$; both), pancreas ($p < 0.001$; both), kidney ($p < 0.001$; both), and spleen ($p < 0.001$; both), and also CAT activity in the testis ($p < 0.001$) (Figures 2 and 3; see Supplementary Materials—Tables S2 and S3 for details). The occurrence of the mentioned interactions indicates that the main effects had no significant influence on the parameters examined or were mutually cancelled out.

3.2.1. Effect of CuNP Dose

The two-way ANOVA revealed that increasing the CuNPs level from 6.5 to 13 mg/kg diet did not significantly impact the redox parameters in blood and tissues (Table 2, Figures 1–3; see Supplementary Materials—Tables S1–S3 for details).

3.2.2. Effect of Fibre Type

Irrespective of the level of CuNPs, feeding rats a diet containing pectin, inulin, or psyllium resulted in a decrease in SOD levels in blood plasma ($p < 0.001$) compared to the control group receiving cellulose as a standard fibre source. Pectin or psyllium inclusion in the diet reduced Cp levels in blood plasma; meanwhile, inulin inclusion increased this indicator ($p < 0.001$) compared to rats receiving a diet containing only cellulose as a fibre source. Pectin or psyllium inclusion into the diet also elevated 3-NT levels ($p = 0.033$) in blood plasma compared to the control group. Furthermore, rats fed a diet supplemented with pectin had reduced PC levels ($p < 0.001$) in blood plasma, and psyllium supplementation to the diet resulted in elevated 8-OHdG levels ($p = 0.028$) in the blood plasma relative to the cellulose-only control group. Psyllium inclusion in the diet also significantly reduced CASP3 levels ($p = 0.017$) in the rats' blood plasma compared to rats from the group that received a pectin-supplemented diet (Table 2). In the hearts of rats receiving an inulin-supplemented diet, a lower MDA level than in the control group was observed (Figure 1; see Supplementary Materials—Table S1 for details).

4. Discussion

The current experiment utilises Wistar rats (Cmdb:Wi) as they are a well-established model for nutritional and metabolic studies, particularly in examining the internal organs' responses to nutritional interventions and the systemic metabolic changes associated with dietary supplementation. It is essential to acknowledge the limitations when extrapolating findings from animal studies to humans. These limitations arise not only from species differences but also from variations in dietary fibre and copper content, as well as the challenges of testing dietary nanoparticles in humans. Nevertheless, a substantial body of the literature supports the notion that the rat model offers significant advantages for investigating human health and disease in relation to dietary habits and environmental factors. The available literature indicates that the CuNPs introduced into the organism may induce oxidative stress [33–35]. The mechanism of the CuNPs' prooxidant action seems to result primarily from the fact that, under the influence of the strong oxidants that occur in living cells, they can undergo oxidation processes to Cu^{+} and Cu^{2+} ions [3,36], which then enter the redox cycle [11,12]. In the presence of reducers such as ascorbic acid or glutathione, Cu^{2+} ions may reduce to Cu^{+} ions which, when reacting with hydrogen peroxide (H_2O_2) in the Fenton reaction, lead to the highly reactive hydroxyl radicals' synthesis. Cu^{+} ions can also reduce molecular oxygen (O_2), forming the superoxide anion radical ($O_2^{\bullet -}$). Importantly, after reacting with H_2O_2 or O_2, Cu^{+} ions re-oxidise to Cu^{2+}, allowing them to re-enter the redox cycle, generating further reactive oxygen species (ROS) portions [11,12]. Due to their high reactivity and low specificity, these species can oxidise lipids, proteins, and nucleic acids, disrupting cellular function by compromising cell membrane integrity and protein functionality and causing DNA mutations that may contribute to cancer development [14,15].

Various antioxidant mechanisms have evolved in living organisms to mitigate the risk of such adverse oxidative changes induced by free radical activity. Among these, endogenous enzymatic antioxidant defence, including enzymes like superoxide dismutase (SOD) and catalase (CAT), plays a crucial role [37]. SOD catalyses the conversion of the aggressive superoxide radical ($O_2^{\bullet -}$) to H_2O_2 and O_2 [21], while CAT neutralises the

resulting H_2O_2 to H_2O and O_2 [38]. The results of our study confirmed that replacing the standard Cu form ($CuCO_3$) with CuNPs in rat diets influenced the enzymatic antioxidant defence, as evidenced by an increase in CAT levels and a simultaneous decrease in SOD levels in the blood. Potentially, this could indicate enhanced free radical synthesis due to the CuNPs' action. The observed reduction in blood SOD levels may suggest the depletion of enzyme resources in response to increased superoxide anion ($O_2^{\bullet -}$) generation in the presence of CuNPs. It is possible that the oxidative degradation of SOD could occur under experimental conditions, reducing its activity and stability, too. The increased CAT level may also indicate enhanced H_2O_2 synthesis due to the replacement of $CuCO_3$ with CuNPs in the rats' diet, which the body tries to combat by adjusting the enzyme activity to the current catalytic needs. However, it is worth noting that the observed changes in blood antioxidant enzyme levels, which suggest potential oxidative stress, were not accompanied by adverse changes in the total antioxidant status (TAS), biomarkers of protein oxidation (PC), lipid (MDA), and DNA (8-OHdG), or elevated markers of apoptotic cell death (CASP-3 and CASP-8). This suggests that the organism may adapt to the recommended level of CuNPs in the diet by modulating antioxidant enzyme activity to maintain proper redox status. Furthermore, the results of our study showed that replacing $CuCO_3$ with CuNPs in the rat diet positively stimulated the antioxidant response in liver and small intestine cells, subsequently reducing lipid peroxidation, as indicated by decreased MDA levels. The small intestine is initially exposed to the highest CuNP concentrations due to direct contact with food content [27], while the liver serves as the main organ responsible for Cu metabolism and storage [39]. Both tissues are characterised by dense vascularisation and high metabolic activity [40,41], which favors exposure to potentially pro-oxidative CuNP action. Therefore, the obtained results seem to confirm that the dietary inclusion of CuNPs in the diet at the recommended dose does not generate excessive oxidative stress in the blood and tissues of rats.

However, it appears concerning that replacing $CuCO_3$ with CuNPs in the rats' diet led to reduced levels of APE-1 and DNA methylation in blood, potentially suggesting their negative impact on DNA stability and integrity. APE-1 (apurinic/apyrimidinic endonuclease 1) is a crucial enzyme in DNA repair through the base excision repair (BER) pathway. BER is initiated by DNA glycosylases, which identify and remove damaged bases, creating apurinic/apyrimidinic (AP) sites in the DNA chain. APE-1 then initiates the repair process by cutting the DNA backbone to remove AP sites and replacing them with correct nitrogenous bases, which helps maintain genome stability [42]. Therefore, the observed reduction in APE-1 levels in this study may suggest impairment of the DNA repair system or the increased degradation of this protein. Although this study did not demonstrate increased DNA oxidation as the increased production of 8-OHdG in the blood, it cannot be ruled out that extending the period of feeding rats with a diet containing CuNPs would lead to the complete inactivation of APE-1 and accumulation of oxidative DNA damage. Moreover, rats fed a diet containing CuNPs instead of $CuCO_3$ showed a decrease in DNA methylation levels in their blood, which may lead to the deregulation of the expression of various genes, including those responsible for an oxidative stress response and DNA repair. The results of our study indicate that replacing the recommended level of $CuCO_3$ in the rat diet with CuNPs does not negatively affect the oxidation of lipids and proteins in the blood and tissues. However, it may weaken DNA repair processes and reduce DNA methylation, significantly increasing the risk of mutations underlying numerous degenerative diseases.

The results of our study did not reveal an effect of doubling the level of CuNPs (13.0 mg Cu/kg diet) compared to nutritional recommendations (6.5 mg Cu/kg diet) in the rat diet on blood redox status indicators. However, it was found that the effect of CuNPs on blood redox status could be modulated by simultaneously introducing alternative dietary

fibre sources. The results of the conducted studies showed a reduction in SOD levels in the blood of rats receiving a diet containing all tested dietary fibre forms—pectin, inulin, or psyllium—regardless of the CuNP level used. Meanwhile, none of these experimental treatments affected CAT activity in rat blood, which could indicate increased oxidative stress. It is hypothesised that the observed decrease in blood SOD levels could be due to a Cu deficiency in the body caused by the inclusion of different dietary fibre forms. Copper is an essential trace element for SOD1 and SOD3 synthesis because it is their cofactor with Zn. Cu's presence in the active center of SOD is crucial for binding the super-oxide anion and its dismutation [43]. Consequently, a lack of Cu in the body may lead to limited SOD synthesis or impaired SOD antioxidant function, increasing the same the risk of oxidative stress and cellular damage. The available literature indicates that the presence of dietary fibre in the diet can decrease the absorption of trace elements, including Cu, from the gastrointestinal tract and impact their bioavailability and metabolism in the body [6,27,44–46]. The study by Krzysik [24] shows that adding pectin or cellulose as dietary fibre sources reduced the absorption of divalent ions from the diet, including Cu. The mechanism by which dietary fibre affects the absorption of Cu from the gastrointestinal tract seems to be closely dependent on the fibre's type and physicochemical properties. The available literature indicates that pectin can chelate divalent ions via free hydroxyl groups, which may, in turn, reduce the amount of Cu available for absorption from the gastrointestinal tract [22,24,47]. Liu [33] and McRorie [48] also report that both pectin and psyllium have high viscosity and form a unique gel in the gastrointestinal tract, which the entrapment of Cu facilitates. This can physically hinder the dietary micronutrients' contact with the absorptive surface of the intestines, thereby reducing Cu absorption [33,48]. Conversely, inulin seems to act oppositely to pectin and psyllium. It is a prebiotic that stimulates the growth of beneficial bacteria in the intestines, whose activity can affect mineral metabolism and absorption. Moreover, inulin fermentation produces short-chain fatty acids (SCFAs) such as butyrate, propionate, and acetate [49,50], which acidify the intestinal contents. Consequently, it may increase Cu solubility, thus enhancing its absorption from the gastrointestinal tract [50]. Nonetheless, irrespective of the type of dietary fibre used, this ingredient can accelerate intestinal transit, reducing the contact time of food contents with enterocytes of the small intestine, which may also limit the number of nutrients and minerals absorbed into the body [24,51]. The results of our previous studies have also confirmed that dietary fibre can limit the bioavailability of dietary Cu [6]. It has been shown that the addition of dietary fibre in the form of pectin, inulin, or psyllium to a diet with twice the CuNP content of dietary recommendations significantly increased Cu excretion and prevented its accumulation in the brain and muscles of rats [6]. In light of the above, it can be assumed that the observed decrease in blood SOD levels in our study may result from a Cu deficiency caused by its reduced absorption from the gastrointestinal tract due to supplementation with the tested alternative forms of dietary fibre. This hypothesis seems to be also supported by the decreased Cp levels in the blood of rats fed a diet with pectin or psyllium addition, regardless of the CuNP level. Ceruloplasmin is a glycoprotein that binds about 95% of Cu in blood plasma. Each molecule of ceruloplasmin can bind up to six atoms of this element. Thus, it is considered the most important blood Cu transport protein [52,53]. Furthermore, ceruloplasmin exhibits antioxidant properties, with effects similar to superoxide dismutase, although slightly weaker but more stable [54]. Ceruloplasmin is primarily synthesised in the liver, though small amounts can also be produced in the brain, placenta, kidneys, adipose tissue, and Sertoli cells [55]. This protein is sensitive to Cu deficiency, which quickly inhibits its synthesis [54]. Therefore, the reduced Cp levels in the blood of rats fed diets containing both CuNPs and pectin or psyllium may indicate a disturbance in Cu homeostasis due to its deficiency. The weakened antioxidant defence and increased oxidative stress of rats under

a diet supplemented with pectin or psyllium seem to be confirmed by the increased level of 3-NT in the blood plasma, indicating the intensification of protein nitration processes. Additionally, the rats receiving psyllium in their diets, regardless of using CuNP levels, exhibited increased blood levels of 8-OHdG, suggesting enhanced nucleic acid oxidation. These observed adverse oxidative changes are likely due to a disturbance in Cu homeostasis, leading to a weakened antioxidant defence. Interestingly, however, inulin inclusions in the rats' diet increased Cp levels in the blood, indicating its potentially beneficial effect on Cu metabolism in the body. This observed effect may be due to the antioxidant properties of inulin and improved Cu absorption in the gastrointestinal tract throughout the acidification of intestinal contents resulting from the fermentation of this type of fibre [54]. Moreover, rats who received a diet containing CuNPs, regardless of the dose, and inulin did not exhibit increased oxidative reactions of lipids, proteins, and nucleic acids in the blood. The conducted studies also showed that inulin inclusion in the diet, irrespective of the CuNPs' supplementation level, reduced MDA levels in the heart. Therefore, it can be assumed that, unlike pectin and psyllium, the presence of inulin in the diet does not negatively affect Cu homeostasis and the blood antioxidant system, thereby enabling the effective protection of cells from oxidative processes.

The results of the redox status analyses of the internal organs conducted in this experiment did not show distinct effects of the main experimental factors, such as increasing the dietary CuNP level from the recommendation (6.5 mg Cu/kg) to twice the nutritional recommendation (13.0 mg Cu/kg) and replacing standard dietary fibre (cellulose) with alternative forms (pectin, inulin, or psyllium) in rat diets. Nevertheless, the obtained results allow us to notice certain relationships between the type of dietary fibre used and the level of CuNPs in the diet, in the context of the redox status of the tested organs. In rats fed a diet containing the recommended CuNP level and alternative dietary fibre, regardless of its form, the stimulation of the enzymatic antioxidant defence response was observed in the studied organs, with the effect being most beneficial in the case of inulin. Our previous research indicates that replacing the recommended level of $CuCO_3$ with CuNPs in rats' diets may intensify lipid peroxidation processes in the lungs and kidneys [17]. However, the current study showed that none of the analysed organs in rats supplemented with inulin and a standard dose of CuNPs in the diet exhibited increased lipid peroxidation. Interestingly, lipid peroxidation in the lungs, pancreas, and testes was even favorably inhibited, as evidenced by decreased MDA levels. In light of the above, it can be concluded that inulin effectively protects the body against the potentially toxic, pro-oxidant effects of CuNPs. In turn, pectin inclusion in the rats' diets containing standard CuNP levels reduced lipid peroxidation in the liver but enhanced this process in the intestine and kidney. Psyllium supplementation in the rats' diet containing a recommended level of CuNPs increased lipid peroxidation in the spleen and liver. Our previous studies indicate that replacing the recommended $CuCO_3$ level with CuNPs in the rats' diet attenuates lipid peroxidation processes in the liver. Therefore, it seems that the simultaneous inclusion of pectin or psyllium in the rat diet containing the recommended level of CuNPs is not sufficient to ensure the effective functioning of the antioxidant system, which may be probably related to the limitation of the amount of Cu absorbed from the gastrointestinal tract due to the strengthening of the intestinal barrier. Interestingly, the situation is markedly different with a twofold increase in dietary CuNPs. The most effective stimulation of antioxidant enzyme activity (SOD and CAT) in internal organs was observed in rats simultaneously receiving psyllium in their diets, which translated into reduced lipid peroxidation levels in the lungs, liver, pancreas, spleen, and testes. Nevertheless, rats from this treatment showed intensified lipid peroxidation in the small intestine, which may be related to the fact that this tissue was the first of all the examined tissues to be exposed to direct contact with the CuNPs

introduced into the body with the diet and at the highest concentration. The elevated CuNP amount in the digestive contents could lead to the increased local concentration of reactive oxygen species, subsequently increasing lipid peroxidation in adjacent tissues. The results of our study indicate that, with excessive CuNPs in the diet, pectin addition could also favorably modulate the antioxidant system, protecting the pancreas and spleen from lipid oxidation. However, it was insufficient to shield the liver from CuNP-generated free radicals, as indicated by elevated MDA levels in this tissue. Among all the alternative fibres tested, inulin demonstrated the least protective effect against the prooxidative effects of excess CuNPs, as it did not reduce lipid peroxidation in any of the tissues examined and even exacerbated this process in the lungs, pancreas, kidneys, liver, and testes. It is highly likely that the improved redox status of the studied tissues through pectin or psyllium supplementation in the diet results from these fibres' role in limiting the amount of CuNPs absorbed from the gastrointestinal tract, thereby reducing the generation of free radicals in the body. There is also a possibility that the antioxidant potential of the applied dietary fibers results from their direct beneficial effect on the gut microbiota composition and the increased production of short-chain fatty acids (SCFAs) during fiber fermentation. The literature data indicate that SCFAs exhibit strong antioxidant properties by enhancing the activity of antioxidant enzymes, inducing protective proteins, and activating the Nrf2–Keap1 signaling pathway [56]. Our previous research also confirmed that the inclusion of experimental types of dietary fiber in diets containing copper nanoparticles (CuNPs) significantly increased the activity of intestinal bacterial enzymes such as α-glucosidase, β-glucosidase, β-glucuronidase, and α-arabinofuranosidase, as well as the production of SCFAs in rats, with the most pronounced effect observed with pectin supplementation [57]. This suggests that functional fiber may mitigate the negative impact of CuNPs on the gut microbiota by supporting its metabolic activity, which in turn may translate into enhanced antioxidant protection of the organism. In turn, lipid oxidation in the tissues of rats receiving excess CuNPs in their diet, enhanced by inulin, is probably because this additive, due to its physicochemical properties, may support the absorption of Cu, or at least to a lesser extent than pectin and psyllium, inhibit its absorption. Consequently, this could increase Cu bioavailability in the body, raising the risk of enhanced reactive oxygen species generation.

5. Conclusions

In conclusion, replacing $CuCO_3$ with CuNPs in the rat diet did not affect protein and lipid oxidation processes, but it deteriorated DNA stability and integrity in the blood by weakening the DNA repair system and reducing DNA methylation levels. However, this treatment favorably limited lipid peroxidation in the liver and small intestine. Regardless of the CuNPs level in the rats' diet, inulin did not negatively impact redox status; meanwhile, pectin intensified protein nitration processes, and psyllium induced both protein nitration and DNA oxidation in the blood. Furthermore, adding pectin or psyllium to rats' diets with standard CuNP content weakened the antioxidant defence of internal organs and intensified oxidative changes in tissues, likely due to the Cu deficiency resulting from increased Cu binding in the gastrointestinal tract. However, in the presence of excessive amounts of CuNPs in the diet, pectin or psyllium inclusion reduced the release of free radicals by CuNPs, thereby beneficially affecting the redox status of tissues. Meanwhile, inulin inclusion improved the redox status of tissues in rats fed the recommended CuNP level in the diet but worsened it when the dietary CuNP level was excessive, likely due to increased Cu absorption from the gastrointestinal tract in the presence of inulin. The obtained results suggest that the addition of dietary fibre in the form of inulin may beneficially stimulate the response of the redox system in the conditions of CuNP nutrition at the recommended

dose, pectin, or psyllium in the case of an excessive supply of CuNPs in the diet. Thus, selecting the appropriate type of dietary fibre based on the CuNP level in the diet may effectively protect the organism from the potentially harmful prooxidative effect of CuNPs, ultimately contributing to the favourable regulation of their metabolic impact in the body.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antiox14060695/s1, Table S1. Level of malondialdehyde (MDA; μmol/g) in selected tissues in rats fed experimental diets; Table S2. Activity of superoxide dismutase (SOD; U/g) in selected tissues in rats fed experimental diets; Table S3. Activity of catalase (CAT; U/g) in selected tissues in rats fed experimental diets.

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Institutional Review Board Statement: All procedures involving animals were carried out following Polish regulations on animal experimentation and ethical standards, and were fully compliant with Directive 2010/63/EU of the European Convention for the Protection of Vertebrate Animals for Experimental and Other Scientific Purposes [29]. The Local Ethics Committee for Animal Experiments in Olsztyn approved the experimental protocol (Approval No. 19/2021; Olsztyn, Poland; Approval date: 17 March 2021).

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Abbreviations

3-NT	3-nitrotyrosine
8-OHdG	8-hydroxy-2′-deoxyguanosine
APE-1	Apurinic/apyrimidinic endonuclease 1
Casp3	Caspase 3
Casp8	Caspase 8
CAT	Catalase
Cp	Ceruloplasmin
CuNPs	Copper nanoparticles
EDTA	Ethylenediaminetetraacetic acid
Group C	Rats fed a control diet with standard Cu content in the mineral mixture (6.5 mg/kg from CuCO3), with 8% of cellulose as a dietary fibre source
Group CH	Rats fed a control diet with enhanced Cu content in the mineral mixture (13 mg/kg from CuCO3), with 8% of cellulose as dietary fibre source
Group CN	Rats fed diets with supplementation of CuNPs (6.5 from Cu-nanoparticles), with 8% of cellulose dietary fibre source
Group CNH	Rats fed diets with supplementation of CuNPs (13 mg/kg from Cu-nanoparticles), with 8% of cellulose dietary fibre source
Group JN	Rats fed diets with supplementation of CuNPs (6.5 mg/kg from Cu-nanoparticles), with 2% of cellulose and 6% of inulin dietary fibre source

Group JNH	Rats fed diets with supplementation of CuNPs (13 mg/kg from Cu-nanoparticles), with 2% of cellulose and 6% of inulin dietary fibre source
Group PN	Rats fed diets with supplementation of CuNPs (6.5 mg/kg from Cu-nanoparticles), with 2% of cellulose and 6% of pectin dietary fibre source
Group PNH	Rats fed diets with supplementation of CuNPs (13 mg/kg from Cu-nanoparticles), with 2% of cellulose and 6% of pectin dietary fibre source
Group SN	Rats fed diets with supplementation of CuNPs (6.5 mg/kg from Cu-nanoparticles), with 2% of cellulose and 6% of psyllium dietary fibre source
Group SNH	Rats fed diets with supplementation of CuNPs (13 mg/kg from Cu-nanoparticles), with 2% of cellulose and 6% of psyllium dietary fibre source
MDA	Malondialdehyde
OGG1	8-oxoguanine DNA glycosylase
PBS	Phosphate-buffered saline
PC	Protein carbonyl derivative
SCFAs	Short-chain fatty acids
SOD	Superoxide dismutase
TAS	Total antioxidant capacity

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

Aleksandra Marzec[1], Ewelina Cholewińska[1], Bartosz Fotschki[2], Jerzy Juśkiewicz[2]♦, Anna Stępniowska[1], Katarzyna Ognik[1]

[1]Department of Biochemistry and Toxicology, Faculty of Animal Sciences and Bioeconomy, University of Life Sciences in Lublin, Akademicka 13, 20-950 Lublin, Poland
[2]Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Tuwima 10, 10-748 Olsztyn, Poland
♦Corresponding author: j.juskiewicz@pan.olsztyn.pl

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 $CuCO_3$. 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 $CuCO_3$ 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 biodistribution. 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 neurodegenerative 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 microparticles (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). $CuCO_3$ 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

[1]Casein 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. [3]AIN-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 B_{12} (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 D_3 (cholecalciferol, 400 000 IU g^{-1}), 0.075 vitamin K_1 (phylloquinone), 974.655 powdered sucrose. [4]In the experimental treatments with CuNPs the MX did not include $CuCO_3$, 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. [5]Maize 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§	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/kg	mg/kg	mg/kg
Control C	0.629	0.631	0.028	0.577	0.605	8.38	4.08	2.21	4.50	1.02
Control CH	1.22	1.22	0.021	0.907	0.928	25.8	24.1	1.75	4.20	0.791
2-way ANOVA										
CN	0.618 c	0.620 cd	0.017 de	0.478 c#	0.496 c#	22.8 c#	19.9 cd#	1.79#	4.10	0.714 cd#
CNH	1.17 a	1.17 a	0.015 e	0.492 c&	0.507 bc&	58.0 a&	56.8 a&	1.74	4.18	0.801 c
PN	0.609 c	0.611	0.028 cde	0.467 c#	0.494 c#	23.7 c#	19.1 cd#	1.71#	4.53	0.564 de#
PNH	1.07 b&	1.07 b&	0.023 cde	0.846 a	0.870 a	20.6 c	18.4 cd	1.63	4.61	0.659 cde
JN	0.556 c	0.558 d	0.034 cd	0.353 d#	0.387 d#	36.6 b#	30.6 b#	1.63#	4.26	0.457 e#
JNH	1.07 b&	1.07 b&	0.061 a&	0.722 b	0.784 a	32.4 b	26.5 bc	1.66	4.19	1.13 b&
SN	0.685 c	0.687 c	0.051 ab	0.548 c	0.599 b	19.9 c#	12.6 d#	1.64#	4.24	1.26 ab
SNH	1.06 b&	1.06 b&	0.040 bc&	0.822 a	0.861 a	22.6 c	18.7 cd	1.60	4.75&	1.33 a&
SEM	0.034	0.034	0.002	0.026	0.027	1.916	1.995	0.032	0.049	0.041
CuNP dose (D)										
L (6.5 mg/kg)	0.613 b	0.615 b	0.032	0.461	0.494	25.3	20.1	1.69	4.28	0.748
H (13 mg/kg)	1.09 a	1.09 a	0.035	0.720	0.755	33.4	30.1	1.66	4.43	0.979
P value	<0.001	<0.001	0.575	<0.001	<0.001	<0.001	<0.001	0.465	0.147	<0.001
Fibre type (F)										
C (cellulose)	0.896	0.897	0.016	0.485	0.501	40.4	38.4	1.76	4.14c	0.758
P (pectin)	0.838	0.840	0.025	0.656	0.682	22.1	18.8	1.67	4.57a	0.612
J (inulin)	0.811	0.813	0.047	0.537	0.585	34.5	28.6	1.64	4.22bc	0.792
S (psyllium)	0.872	0.873	0.045	0.684	0.730	21,2	15.7	1.62	4.50ab	1.29
P value	0.058	0.058	<0.001	<0.001	<0.001	<0.001	<0.001	0.237	0.009	<0.001
Interaction D×F										
P value	0.061	0.062	0.006	<0.001	<0.001	<0.001	<0.001	0.867	0.202	<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 $CuCO_3$, 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=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 pectin as dietary fibre; J, 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, PN, PNH: 7.32, 14.8, 7.33, 13.33, 7.25, 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 $CuCO_3$, 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 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, 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=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 ($Cu(OH)_2$), 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 psyllium, 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 micronutrients 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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ORIGINAL PAPER

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Is the impact of copper nanoparticles on the immune system of rats dependent on the diverse physiological functions of dietary fibre?

A. Marzec[1], B. Fotschki[2], **E. Cholewińska[1], W. Dworzański[3,4], J. Juśkiewicz[2]** and K. Ognik[1,*]

[1] University of Life Sciences in Lublin, Faculty of Animal Sciences and Bioeconomy, Department of Biochemistry and Toxicology, Akademicka 13, 20-950 Lublin, Poland
[2] Polish Academy of Sciences, InLife Institute of Animal Reproduction and Food Research, Trylińskiego 18, 10-683 Olsztyn, Poland
[3] Casimir Pulaski Radom University, Faculty of Medical and Health Sciences, Department of Non-Procedural Clinical Sciences, Bolesława Chrobrego 27, 26-600 Radom, Poland
[4] Casimir Pulaski Radom University, Faculty of Medical and Health Sciences, Clinical Department of Cardiology with Electrotherapy Laboratory, Juliana Aleksandrowicza 5, 26-617 Radom, Poland

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* Corresponding author:
e-mail: katarzyna.ognik@up.edu.pl

ABSTRACT. A six-week feeding trial was conducted to evaluate the effects of dietary supplementation with copper nanoparticles (CuNPs) administered at 6.5 or 13.0 mg Cu/kg diet, in combination with different types of dietary fibre, on haematological and immune parameters in rats. Ten experimental groups were included. Two control groups received cellulose-based diets containing copper(II) carbonate($CuCO_3$) at 6.5 or 13.0 mg Cu/kg diet. In the experimental groups, $CuCO_3$ was replaced with CuNPs at the corresponding concentrations, and diets were supplemented with various fibre types: cellulose, pectin, inulin, or psyllium. At the end of the experimental period, blood samples were collected for the assessment of haematological and immune indices. Irrespective of Cu form or dose, diets containing pectin or inulin lowered the counts of white blood cells (WBC) and lymphocytes (LYM), while inulin or psyllium reduced interleukin 6 (IL-6) concentrations. In contrast, CuNPs administered at 6.5 mg Cu/kg diet in combination with psyllium increased WBC and LYM counts. The inclusion of inulin or psyllium in diets containing the lower CuNPs dose decreased immunoglobulin M (IgM), IL-6, and tumour necrosis factor α (TNF-α) levels. Replacing $CuCO_3$ with CuNPs at 6.5 mg Cu/kg in diets containing cellulose, inulin, or psyllium lowered immunoglobulin A (IgA) levels, while cellulose additionally increased C-reactive protein (CRP) concentrations. A diet containing double dose of CuNPs (13.0 mg Cu/kg) with psyllium reduced mean corpuscular haemoglobin (MCH) and increased red cell distribution width (RDWc) and mean platelet volume (MPV). Moreover, high-dose CuNPs diets containing pectin, inulin, or psyllium lowered IgA, IgM, interleukin 2 (IL-2), and TNF-α concentrations, and pectin additionally reduced immunoglobulin G (IgG) levels. In summary, replacing standard $CuCO_3$ with CuNPs, even at 6.5 mg Cu/kg diet, induced inflammation and impaired immune function in rats. However, supplementation with pectin or inulin alleviated the adverse immune and inflammatory effects caused by Cu nanoparticles.

Introduction

Copper (Cu) is an essential dietary element for both humans and animals. It performs multiple physiological functions, including acting as a cofactor for numerous metabolic enzymes, participating in respiratory chain energy production, neutralising free radicals, as well as maintaining proper connective tissue structure and nerve conduction (Angelova et al., 2011; Opazo et al., 2014; Bost et al., 2016; Tishchenko et al., 2016). The recommended dietary allowance (RDA) for Cu in human adults is 900 μg/day, and the tolerable upper intake level (UL) is 10000 μg/day (10 mg/day), established based on liver damage as the critical adverse effect (NIH, 2025). Substantial evidence indicates that Cu also plays an important regulatory role in immune homeostasis and inflammatory responses (Stafford et al., 2013; Cheng et al., 2022; Liu et al., 2022; Lan et al., 2024; Li et al., 2024; Lu et al., 2024). Recent studies have demonstrated that Cu directly binds to alpha kinase 1 (ALPK1), a cytosolic pattern recognition receptor, leading to its activation. This interaction enhances innate immune signalling via the nuclear factor kappa B (NF-κB) pathway and stimulates the production of pro-inflammatory cytokines, including interleukin 8 (IL-8), interleukin 1β (IL-1β), and tumour necrosis factor α (TNF-α) (Lu et al., 2024).

Cu deficiency has been shown to seriously impair both innate and adaptive immunity. It has been associated with reduced neutrophil counts, excessive suppression of reactive oxygen species (ROS) production, unfavourable alterations in T lymphocyte populations, in particular, a decrease in CD4+ helper T cells, and impaired IL-2 synthesis (Cheng et al., 2022; Lan et al., 2024). Cu is also required to maintain macrophage antimicrobial activity. Pro-inflammatory signals promote Cu accumulation in phagolysosomes, where it supports ROS generation necessary for pathogen elimination (Stafford et al., 2013). Furthermore, Cu acts as a cofactor of superoxide dismutase, a key antioxidant enzyme; its deficiency increases oxidative stress and activates inflammatory pathways (Li et al., 2024). During infection, elevated serum Cu levels have also been observed, accompanied by increased ceruloplasmin activity, which represents an important component of the acute-phase immune response (Li et al., 2024). Mice lacking the ceruloplasmin gene were found to be highly susceptible to bacterial infections and displayed a diminished cytokine response (Liu et al., 2022). Collectively, these findings demonstrate that an optimal Cu status is indispensable for effective immune responses, whereas deficiency leads to significant immunosuppression and increased susceptibility to infections.

Traditionally, inorganic Cu salts such as copper(II) carbonate ($CuCO_3$) have been used as dietary supplements; however, their bioavailability is relatively low (Ognik et al., 2016). In recent years, Cu nanoparticles (CuNPs) have been proposed as a more efficient source of this element. They are more readily absorbed from the gastrointestinal tract compared to $CuCO_3$ and have been shown to positively stimulate immune system responses (Cholewińska et al., 2018). Moreover, CuNPs more effectively inhibit protein oxidation and nitration, thereby preventing protein degradation, limiting DNA methylation (Ognik et al., 2019), and improving the antioxidant status of the liver and brain (Ognik et al., 2020). Despite these potential advantages, the use of CuNPs as dietary supplements is not without risks, particularly in relation to immune and inflammatory processes. Their higher bioavailability may be associated with stronger cytotoxic effects compared to traditional Cu salts. Tulinska et al. (2022) demonstrated that CuO nanoparticles significantly increased T lymphocyte proliferation and the production of both Th1 (IFN-γ, IL-12p70) and Th2 cytokines (IL-4, IL-5), while concurrently suppressing granulocyte phagocytic activity and reducing glutathione levels in mice. In addition, the 'Trojan horse' mechanism attributed to Cu nanoparticles, involving endocytic uptake and subsequent dissolution in lysosomes, leads to intracellular Cu overload, reaching millimolar concentrations in both the cytoplasm and the nucleus. This may induce extensive alterations in the expression of genes related to oxidative stress, DNA damage responses, and inflammatory pathways (Strauch et al., 2017). Our previous study (Cholewińska et al., 2018) has demonstrated that, owing to their antimicrobial properties, Cu nanoparticles markedly reduce the enzymatic activity of beneficial gut bacteria and decrease the production of short-chain fatty acids, which may impair the function of gut-associated lymphoid tissue. Considering all these factors, further research on the regulation of Cu absorption, particularly in nanoparticle form, appears to be essential.

Dietary fibre represents another crucial dietary component, defined as various plant-derived substances that are resistant to digestion by enzymes of human and monogastric animal digestive tracts. Many studies in rats have demonstrated that fibre supplementation, particularly in high-fat diets, improves lipid profiles and limits body weight gain, resulting in values comparable to those observed

in animals fed low-fat diets (Artiss et al., 2006; Lecumberri et al., 2007). Beneficial effects of dietary fibre on gut microbiota composition and maintenance of the intestinal barrier have also been reported (Lee et al., 2015). Different types of dietary fibre significantly influence the pH in the lumen of the small and large intestines, e.g., by increasing water-binding capacity or digesta viscosity. These changes improve gut acidity and may consequently influence Cu absorption (Aggett and Fairweather-Tait, 1998). Fermentation of soluble dietary fibre by the gut microbiota leads to the production of short-chain fatty acids (SCFAs), which lower the pH in the colon and, to a lesser extent, in the ileum. Higher acidity increases Cu solubility and ionisation, thereby facilitating its intestinal absorption. Conversely, a more alkaline environment, frequently associated with low intake of fermentable fibre, may cause Cu to precipitate in the form of insoluble hydroxides, reducing its bioavailability (Wu et al., 2021). Certain types of dietary fibre, particularly those rich in phytates and polyphenols, may also chelate Cu ions and form insoluble complexes, directly inhibiting Cu absorption in the intestinal lumen. In addition, viscous and gelling fibres, such as pectin, increase digesta viscosity and may physically hinder the interaction of Cu ions with transport proteins located on the surface of enterocytes. The increased water-holding capacity of dietary fibre slows gastric emptying and intestinal passage, potentially prolonging or modifying the period available for Cu absorption (Baye et al., 2017; Cholewińska et al., 2023). Studies have also indicated that manipulating dietary fibre content can also indirectly influence Cu bioavailability by altering the intestinal availability of mineral antagonists (Baye et al., 2017). Dietary fibre can alter the bioavailability of minerals such as zinc, calcium, and iron, which share identical or overlapping transport pathways with Cu and may compete for binding sites on mucosal transporters. Moreover, the form of fibre, its fermentability, and degree of polymerisation have been shown to determine both the magnitude and the direction of its effect on the absorption of Cu and other trace elements (Wapnir, 1998; Baye et al., 2017). Thus, the combination of CuNPs with different types of dietary fibre represents a promising direction in research on immune system modulation, as it utilises synergistic potential to limit toxicity while supporting beneficial immune responses. CuNPs, owing to their higher bioavailability compared to conventional Cu salts, suppress the enzymatic activity of the gut microbiota and reduce the production of SCFAs. However, our previous studies have demonstrated that these effects are strongly reduced by functional dietary fibres such as inulin, pectin, and psyllium (Juśkiewicz et al., 2024). As in our previous studies, four different dietary fibre compounds were evaluated, representing distinct functional classes, i.e., cellulose, pectin, inulin, and psyllium. Cellulose was used as a control inert fibre. Turnlund et al. (1985) demonstrated that dietary α-cellulose does not reduce Cu absorption, which provided the rationale for selecting this fibre type as a control. Pectin represents a viscous and gelling fibre, inulin is a prebiotic stimulating the gut microbiota, while psyllium is a bulking fibre that increases stool mass. Our earlier findings indicated that these different fibre types interact differently with CuNPs, which may significantly affect immune system function (Cholewińska et al., 2023; Juśkiewicz et al., 2024; Marzec et al., 2025). Among the fibres tested, inulin was the most effective in restoring butyrate and propionate production even in the presence of CuNPs, which may support the formation of an anti-inflammatory microenvironment through increased SCFA-mediated immunomodulation. In contrast, pectin exhibited the strongest capacity to rapidly increase bacterial enzyme activity and sustain beneficial microbial metabolic functions. Meanwhile, psyllium reduced ammonia formation and the production of putrefactive SCFAs, thereby minimising intestinal inflammatory load (Juśkiewicz et al., 2024). The protective effects of dietary fibre also involve reinforcement of intestinal barrier integrity through increased expression of tight junction proteins and reduced oxidative stress (Cholewińska et al., 2023). In addition, dietary fibre supplementation modifies the kinetics of CuNPs absorption, facilitating controlled release and reducing systemic toxicity, while simultaneously preserving the selective antimicrobial activity of nanoparticles. This activity preferentially targets pathogenic bacteria while protecting lactic acid bacteria (*Lactobacillus*) and other commensals (Lamas et al., 2020; Juśkiewicz et al., 2024). It was therefore hypothesised that dietary supplementation with different fibre types, in combination with CuNPs, would create a unique immunomodulatory environment, enabling the safe and targeted action of CuNPs on the immune system and inflammatory processes. Current evidence regarding the precise *in vivo* immunomodulatory effects of specific fibre types, administered together with Cu nanoparticles, remains limited, particularly in relation to their interaction with the systemic immune response. Previous studies have focused primarily on the separate effects of CuNPs or dietary fibre, or have not comprehensively addressed their synergistic or antagonistic interactions

affecting immune function and metabolic outcomes during co-administration. Expanding this body of evidence is essential for the development of dietary strategies that safely utilise the enhanced bioactivity of Cu nanoparticles while minimising potential health risks.

Based on experimental evidence from animal models, dietary fibres protect against CuNPs-induced oxidative damage and inflammation, primarily by improving intestinal barrier function. However, indirect mechanisms involving modulation of the gut microbiota or systemic immune responses cannot be excluded. Cholewińska et al. (2023) observed that bioactive pectin- and fructan-type fibres strengthened the intestinal barrier, most likely by increasing mucus viscosity, intestinal content mass, and the expression of protective barrier proteins (e.g., ZO-1). These fibres also reduced CuNPs absorption, presumably by binding Cu ions in the gut lumen and limiting their uptake. The gut microbiota has been identified as a key regulator of intestinal immunity under exposure to nanomaterials, and ingested metal nanoparticles markedly alter the enzymatic and metabolic activity of large-intestinal microorganisms (Tang et al., 2021; Cholewińska et al., 2023; Juśkiewicz et al., 2024; Marzec et al., 2025). Therefore, it was hypothesised that dietary CuNPs supplementation, combined with a neutral control substance (cellulose), a prebiotic (inulin), a viscous fibre (pectin), or a bulking fibre (psyllium), would influence physiological responses and thereby regulate the immunological effects of CuNPs. The objective of the study was to determine whether the inclusion of different types of dietary fibre, i.e., inulin, pectin, or psyllium, in diets containing the lower or a twofold higher dose of CuNPs (6.5 or 13.0 mg Cu/kg) would improve immune function.

Material and methods

The present study is a part of a broader research initiative aimed at investigating the impact of dietary CuNPs in combination with different types of dietary fibre (cellulose, inulin, pectin, and psyllium) on various aspects of the biological response in rats. Accordingly, the experimental design and methodological procedures have been described in detail in studies previously published by the authors (Cholewińska et al., 2023; Majewski et al., 2023; Juśkiewicz et al., 2024; Marzec et al., 2024; 2025).

Copper nanoparticles and dietary fibre

Cu nanoparticles (Cu^0) were purchased from Sky Spring Nanomaterials, Inc. (Houston, TX, USA). The material was supplied by the manufacturer as a nanopowder with 99.9% purity, a nominal particle size of 40–60 nm, and a predominantly spherical morphology. The reported bulk density was 0.19 g/cm³, while the true density was 8.9 g/cm³. The experiment used the same metallic CuNPs as in earlier studies by the authors (Ognik et al., 2016; Cholewińska et al., 2018; Ognik et al., 2019; Ognik et al., 2020; Cholewińska et al., 2023; Majewski et al., 2023; Juśkiewicz et al., 2024; Marzec et al., 2024; Marzec et al., 2025), and their physicochemical properties were previously characterised in detail by Cholewińska et al. (2018). $CuCO_3$, used as a control dietary source of Cu, was obtained from Merck KGaA (Darmstadt, Germany). A control dietary fibre source was used in the form of α-cellulose (Sigma, Poznań, Poland). The experimental dietary fibre compounds used in the experiment were: pectin (PectinE 440(I), Brouwland, Beverlo, Belgium), inulin (Frutafit Tex, Sensus, Netherlands), and psyllium (Psyllim husk powder, NaturaleBio, Rome, Italy).

Experimental protocol

All animal care and experimental procedures complied with Polish legislation concerning animal experimentation and ethical standards, as well as with the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes and Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. The study protocol was approved by the Local Ethics Committee for Animal Experiments in Olsztyn (Approval No. 19/2021; Olsztyn, Poland).

The animals were obtained from the laboratory rat breeding facility (breeder register No. 051) at the Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences (IARFR PAS) in Olsztyn, Poland. Healthy 9-week-old outbred male Wistar rats (Cmdb:Wi CMDB) were fed for 6 weeks a standard semi-purified rat diet containing two levels of CuNPs (6.5 and 13 mg/kg diet, respectively) in combination with different types of dietary fibre. All diets were prepared in the laboratory using high-quality components, including casein as the main protein source, rapeseed oil as the fat source, and maize starch as the main energy source (Table 1). The control dietary fibre, α-cellulose, was added at 8% of the diet, while the experimental fibres, inulin (prebiotic), psyllium (bulking), and pectin (viscous), were added at 6% of the diet in place of cellulose. Depending on the dietary treatment, the corresponding dietary fibres were added as dry powdered preparations directly to the diet formulations.

Table 1. The composition of experimental diets administered to rats for 6 weeks (diet composition: % (g/100 g))

Indices	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

[1] casein preparation: %: crude protein 89.7, crude fat 0.3, ash 2.0, water 8.0; [2] α-cellulose (SIGMA, Poznan, Poland), the main source of dietary fibre; [3] AIN-93G-VM (Reeves, 1997), g/kg mix: nicotinic acid 3.0, Ca pantothenate 1.6, pyridoxine-HCl 0.7, thiamine-HCl 0,.6 riboflavin 0.6, folic acid 0.2, biotin 0.02, vitamin B_{12} (cyanocobalamin, 0.1% in mannitol), 15.0 vitamin E (all-rac-α-tocopheryl acetate, 500 IU/g), vitamin A 0.8 (all-trans-retinyl palmitate, 500000 IU/g), vitamin D_3 0.25 (cholecalciferol, 400000 IU/g), vitamin K_1 0.075 (phylloquinone), powdered sucrose 974.655; [4] in the experimental treatments with CuNPs in the MX $CuCO_3$ was not included. For the safety of the technician preparing the experimental diets, the CuNPs preparation was added as an emulsion with dietary rapeseed oil. This procedure has been successfully used in our previous experiments; [5] maize starch preparation: %: crude protein 0.6, crude fat 0.9, ash 0.2, total dietary fibre 0, water 8.8; groups C and CH – fed a control diet with tested and enhanced Cu content in the mineral mixture (6.5 and 13 mg/kg from $CuCO_3$, respectively) with 8% of cellulose as dietary fibre source; groups CN and CNH – fed diets with supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 8% of cellulose dietary fibre source; groups PN and PNH – fed diets with supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of pectin dietary fibre source; groups JN and JNH – fed diets with supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of inulin dietary fibre source; groups SN and SNH – fed diets with supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of psyllium dietary fibre source

The control diet contained a mineral mixture providing Cu at either the basal dose (6.5 mg/kg diet) or the twofold higher dose (13 mg/kg diet), with $CuCO_3$ as the Cu source. Experimental diets containing the conventional Cu form $CuCO_3$ were prepared by thoroughly mixing appropriately weighed amounts of the salt with the mineral premix, which was subsequently incorporated into the basal diet. For experimental diets containing CuNPs (6.5 and 13 mg/kg diet), direct addition to the mineral premix was not feasible due to the fine particulate nature of the material. Instead, after weighing, the nanoparticles were dispersed in an appropriate amount of canola oil, a component of the basal diet, to prevent loss of this micronutrient. The resulting suspension was then added to the basal diets previously mixed with the mineral premix containing the remaining essential trace elements. All components were subsequently thoroughly mixed to ensure uniform nanoparticle distribution throughout the entire batch. Directly before feeding, each diet was remixed to maintain homogeneity. The average dietary intake in the experimental groups during the experimental period ranged from 17.9 to 19.1 g per animal per day. Detailed intake data have been reported previously (Cholewińska et al., 2023). The experimental design comprised 10 groups, 10 animals each.

Sample collection and analyses

Before the end of the experiment, the rats were fasted for 8 h with free access to water. Next, they were anaesthetised by intraperitoneal injection of ketamine (K) and xylazine (X) in 0.9% NaCl (100 and 10 mg/kg body weight, respectively), in accordance with guidelines for anaesthesia and euthanasia of laboratory rodents. Following laparotomy, blood samples were collected from the caudal vena cava into heparinised tubes and EDTA-coated tubes. The animals were subsequently euthanised by cervical dislocation following Annex IV to Directive 2010/63/EU (n = 10 per group). Blood plasma was obtained by allowing whole blood collected into heparinised tubes to clot, followed by low-speed centrifugation (350 *g*, 10 min, 4 °C). Plasma samples were stored at −80 °C until analysis.

In whole blood, the following haematological parameters were determined using an ABACUS Jr VET Analyzer (DIATRON MI PLC, Budapest, Hungary): total white blood cell (WBC) count,

lymphocyte (LYM) count and percentage, medium-sized cell (MID) count and percentage, neutrophils (NEU) count and percentage, red blood cell count (RBC), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDWc), platelet (PLT) count, platelet percentage (PCT), mean platelet volume (MPV), and platelet distribution width (PDWc). In plasma, the concentrations of selected immune parameters were measured: immunoglobulins A, G, and M (IgA, IgG, and IgM), interleukin-6 (IL-6), interleukin-2 (IL-2), tumour necrosis factor a (TNF-α), and C-reactive protein (CRP). These measurements were performed using commercial enzyme-linked immunosorbent assay (ELISA) kits (MyBioSource Inc., San Diego, CA, USA), strictly following the manufacturer's instructions without modifications. Absorbance was read at 450 nm using a SunriseTM ELISA reader (Tecan Group Ltd., Männedorf, Switzerland). Plasma albumin (ALB) concentration was determined using an automatic biochemical analyser (Plasma Diagnostic Instruments Horiba, Kyoto, Japan).

Statistical analysis

Data were analysed using STATISTICA, version 12.0 (StatSoft Corp., Krakow, Poland) to determine whether variables differed between treatment groups. A two-way ANOVA was applied to assess the effects of the main factors, i.e., CuNPs inclusion level (L, 6.5 mg/kg and H, 13 mg/kg) and dietary fibre type (cellulose, pectin, inulin, and psyllium), as well as their interactions. When ANOVA indicated significant treatment effects, group means were compared using Duncan's multiple-range test. In addition, each experimental group receiving the lower level of CuNPs (L) was compared with the corresponding control group (C; diet containing 6.5 mg/kg Cu from $CuCO_3$ with cellulose as the main fibre) using a t-test. Experimental groups receiving the higher CuNPs dose (H) were similarly compared with the corresponding control group (CH; diet containing 13 mg/kg Cu from $CuCO_3$ with cellulose) using a t-test. Data were checked for normality prior to analysis, and differences were considered significant at $P \leq 0.05$.

Results

Comparison of CN, PN, JN, SN vs. C group

The experimental groups receiving the lower CuNPs dose (6.5 mg/kg) were compared with the corresponding C group – 6.5 mg/kg Cu from $CuCO_3$ and 8% cellulose as the dietary fibre source. The CN group was fed a diet supplemented with 6.5 mg/kg CuNPs and 8% cellulose as the dietary fibre source. In the PN group, the diet contained 6.5 mg/kg CuNPs, with 2% cellulose and 6% pectin as the fibre source. The JN group received 6.5 mg/kg CuNPs with 2% cellulose and 6% inulin, while the SN group received 6.5 mg/kg CuNPs with 2% cellulose and 6% psyllium.

Significant differences between groups were assessed using one-way ANOVA ($P = 0.05$). Rats in the SN group showed higher WBC and lymphocyte counts in the blood (Table 2). Compared to the C group, rats in the JN and SN groups had lower plasma concentrations of IgM, IL-6, and TNF-α. On the other hand, IgA levels were reduced in the CN, JN, and SN groups relative to the control. IL-2 concentrations were elevated in the CN and PN groups compared with the C group. CRP levels were increased in the CN group and decreased in the PN group relative to the control (Table 4).

Comparison of CNH, PNH, JNH, SNH vs. CH group

The experimental groups receiving the higher CuNPs dose (13.0 mg/kg) were compared with the corresponding CH control group, fed a diet containing 13.0 mg/kg Cu from $CuCO_3$ and 8% cellulose as the dietary fibre source. The CNH group was fed a diet supplemented with 13.0 mg/kg CuNPs and 8% cellulose. In the PNH group, the diet contained 13.0 mg/kg CuNPs with 2% cellulose and 6% pectin. The JNH group received 13.0 mg/kg CuNPs with 2% cellulose and 6% inulin, while the SNH group was administered 13.0 mg/kg CuNPs with 2% cellulose and 6% psyllium.

Compared to the CH group, rats in the SNH group showed a decrease in mean corpuscular haemoglobin (MCH) and an increase in red cell distribution width (RDWc) and mean platelet volume (MPV) (Table 3). Plasma levels of IgA, IgM, IL-2, and TNF-α were lower in the PNH, JNH, and SNH groups compared to the control CH group. IgG concentrations were reduced in the PNH group, while CRP levels were decreased in the CNH group relative to CH group.

Two-way ANOVA

Two-way analysis of variance showed that, compared to cellulose (standard fibre), supplementation with pectin (P) or inulin (J) reduced WBC and LYM counts in rat blood ($P = 0.015$ and $P = 0.011$, respectively; Table 2). No significant effects of the

Table 2. White blood cell parameters in the blood of rats fed experimental diets (n = 10 per group)*

Indices	WBC, $10^3/\mu l$	LYM, $10^3/\mu l$	MID, $10^3/\mu l$	NEU, $10^3/\mu l$	LYM, %	MID, %	NEU, %
Control C	5.73	4.61	0.310	0.811	80.8	5.32	14.2
Control CH	5.75	4.51	0.366	0.872	78.3	6.27	15.4
2-way ANOVA:							
CN	6.86	5.50	0.393	0.970	80.5	5.54	14.0
CNH	6.45	5.27	0.272	0.908	81.3	4.37	14.2
PN	5.65	4.55	0.294	0.808	80.3	5.34	14.4
PNH	5.96	4.71	0.397	0.849	79.0	6.65	14.3
JN	5.60	4.48	0.273	0.845	79.4	5.32	15.0
JNH	5.91	4.66	0.410	0.843	78.4	7.22	14.4
SN	6.69$^{\#}$	5.56$^{\#}$	0.318	0.815	83.1	4.73	12.2
SNH	6.64	5.35	0.287	1.00	80.4	4.17	15.5
SEM	0.115	0.103	0.019	0.027	0.599	0.310	0.386
CuNPs dose (D)							
L (6.5 mg/kg)	6.20	5.02	0.320	0.860	80.8	5.23	13.9
H (13 mg/kg)	6.26	5.00	0.344	0.903	79.7	5.64	14.6
P-value	0.811	0.979	0.574	0.461	0.432	0.567	0.406
Fibre type (F)							
C (cellulose)	6.65^{a}	5.39^{a}	0.333	0.939	80.9	4.96	14.1
P (pectin)	5.80^{b}	4.63^{b}	0.346	0.829	79.6	6.00	14.4
J (inulin)	5.80^{b}	4.60^{b}	0.347	0.849	78.8	6.34	14.7
S (psyllium)	6.67^{a}	5.46^{a}	0.303	0.909	81.7	4.45	13.8
P-value	0.015	0.011	0.881	0.520	0.451	0.205	0.906
Interaction D×F							
P-value	0.675	0.842	0.127	0.497	0.846	0.332	0.374

* dietary treatments used in the experimental feeding period: groups C and CH – fed a control diet with tested and enhanced Cu content in the mineral mixture (6.5 and 13 mg/kg from $CuCO_3$, respectively) with 8% of cellulose as dietary fibre source; groups CN and CNH – fed diets with supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 8% of cellulose dietary fibre source; groups PN and PNH, fed diets with supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of pectin dietary fibre source; groups JN and JNH – fed diets with supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of inulin dietary fibre source; groups SN and SNH – fed diets with supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of psyllium dietary fibre source; L – treatment (n = 40) with dietary CuNPs 6.5 mg/kg dose; H – treatment (n = 40) with dietary CuNPs 13 mg/kg dose; C – treatment (n = 20) with cellulose as dietary fibre; P – treatment (n = 20) with pectin as dietary fibre; J – treatment (n = 20) with inulin as dietary fibre; S – treatment (n = 20) with psyllium as dietary fibre; WBC – total white blood cells, LYM – lymphocytes, MID – mid-sized cells, NEU – neutrophils, SEM – pooled standard error of mean (standard deviation for all rats divided by the square root of rat number, n = 100); ^{ab} – Mean values within a column with unlike superscript letters are shown to be significantly different ($P < 0.05$); differences among the 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 6.5 mg/kg (CN, PN, JN, SN) was compared with the control C one with the aid of t-test ($^{\#}$ indicates a significant difference versus the C group); similarly, each experimental group fed CuNPs 13 mg/kg (CNH, PNH, JNH, SNH) was compared with the control CH one with the aid of t-test ($^{\&}$ indicates a significant difference versus the CH group) - brak w tabeli

main factors, i.e., dietary fibre type or CuNPs dose, were found on red blood cell and platelet indices (Table 3). Inclusion of inulin (J) or psyllium (S) as the dietary fibre source in rats led to a decrease in plasma IL-6 levels ($P = 0.001$) relative to cellulose, while no such effect was observed with pectin supplementation (Table 4).

Two-way ANOVA revealed significant dose × fibre (D×F) interactions for IgM ($P = 0.008$), IgG ($P = 0.032$), IgA ($P < 0.001$), IL-2 ($P < 0.001$), TNF-α ($P = 0.004$), and CRP ($P < 0.001$), indicating that the main factors: dietary fibre type (F) and CuNPs dose (D), did not have a uniform effect on these parameters. For IgM, the D×F interaction resulted from the fact that an increased CuNPs dose, combined with cellulose or pectin supplementation, decreased IgM levels, whereas no change was observed with inulin or psyllium. For IgG and TNF-α, the interaction results showed that only the combination of a higher CuNPs dose with pectin

Table 3. Red blood cell and platelet parameters in the blood of rats fed experimental diets (n = 10 per group)*

Indices	RBC, 10^6/µl	HGB, g/dl	HCT, %	MCV, fL	MCH, pg	MCHC, g/dl	RDWc, %	PLT, 10^3/µl	PCT, %	MPV, fL	PDWc, %
Control C	9.06	14.6	42.6	47.0	16.1	34.2	18.9	592	0.478	8.08	35.6
Control CH	9.25	14.9	40.0	46.4	16.2	34.7	18.9	598	0.479	8.04	35.8
2-way ANOVA:											
CN	9.14	14.8	42.6	46.7	16.2	34.8	19.0	596	0.442	8.23	36.3
CNH	9.25	14.6	42.8	46.7	15.9	34.1	19.0	591	0.484	8.21	36.4
PN	9.23	14.7	43.0	46.7	15.9	34.2	19.0	620	0.507	8.16	36.1
PNH	9.36	14.8	42.7	46.2	16.0	34.5	19.2	642	0.531	8.31	35.7
JN	9.23	14.7	43.5	47.1	16.0	33.9	19.3	631	0.500	8.33	36.1
JNH	9.30	14.9	43.8	46.9	16.0	34.0	18.9	641	0.449	7.95	35.7
SN	8.92	14.5	42.1	47.2	16.3	34.2	18.9	601	0.494	8.23	35.6
SNH	9.31	14.6	42.6	45.6	15.7[&]	34.2	19.3[&]	625	0.528	8.44[&]	36.5
SEM	0.043	0.062	0.328	0.132	0.055	0.101	0.056	7.096	0.008	0.039	0.109
CuNPs dose (D)											
L (6.5 mg/kg)	9.13	14.7	42.8	46.9	16.1	34.3	19.0	612	0.486	8.24	36.0
H (13 mg/kg)	9.32	14.8	43.0	46.3	15.9	34.3	19.1	626	0.497	8.22	36.1
P-value	0.055	0.490	0.590	0.066	0.094	0.997	0.530	0.417	0.571	0.822	0.815
Fibre type (F)											
C (cellulose)	9.19	14.7	42.7	46.7	16.1	34.4	19.0	593	0.463	8.22	36.3
P (pectin)	9.29	14.7	42.9	46.5	15.9	34.3	19.1	631	0.519	8.23	35.9
J (inulin)	9.30	14.9	43.8	47.0	16.0	34.0	19.1	638	0.472	8.12	35.9
S (psyllium)	9.12	14.5	42.3	46.4	16.0	34.2	19.1	613	0.511	8.34	36.1
P-value	0.500	0.344	0.104	0.518	0.863	0.564	0.941	0.227	0.101	0.401	0.586
Interaction D×F											
P-value	0.679	0.573	0.882	0.269	0.114	0.290	0.200	0.916	0.252	0.061	0.222

dietary treatments used in the experimental feeding period: groups C and CH – fed a control diet with tested and enhanced Cu content in the mineral mixture (6.5 and 13 mg/kg from $CuCO_3$, respectively) with 8% of cellulose as dietary fibre source; groups CN and CNH – fed diets with supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 8% of cellulose dietary fibre source; groups PN and PNH – fed diets with supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of pectin dietary fibre source; groups JN and JNH – fed diets with supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of inulin dietary fibre source; groups SN and SNH – fed diets with supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of psyllium dietary fibre source; L – treatment (n = 40) with dietary CuNPs 6.5 mg/kg dose; H – treatment (n = 40) with dietary CuNPs 13 mg/kg dose; C – treatment (n = 20) with cellulose as dietary fibre; P – treatment (n = 20) with pectin as dietary fibre; J – treatment (n = 20) with inulin as dietary fibre; S – treatment (n = 20) with psyllium as dietary fibre; RBC – red blood cells, HGB – haemoglobin, HCT – haematocrit, MCV – mean corpuscular volume, MCH – mean corpuscular haemoglobin; MCHC – mean corpuscular hemoglobin concentration, RDWc – red cell distribution width, PLT – platelet count, PCT – platelet percentage, MPV – mean platelet volume, PDWc – platelet distribution width, SEM – pooled standard error of mean (standard deviation for all rats divided by the square root of rat number, n = 100); [ab] – mean values within a column with unlike superscript letters are shown to be significantly different ($P < 0.05$); differences among the 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 6.5 mg/kg (CN, PN, JN, SN) was compared with the control C one with the aid of t-test ([#] indicates a significant difference versus the C group); similarly, each experimental group fed CuNPs 13 mg/kg (CNH, PNH, JNH, SNH) was compared with the control CH one with the aid of t-test ([&] indicates a significant difference versus the CH group)

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reduced these parameters, while other fibre types showed no effect. For IgA, the decrease occurred when the higher CuNPs dose was combined with pectin or inulin, but not with cellulose or psyllium. Regarding IL-2, the D×F interaction was due to an increase in IL-2 levels when higher CuNPs dose was combined with cellulose or psyllium, a decrease with pectin, and no effect with inulin. For CRP, the higher CuNPs dose increased CRP levels when combined with pectin, decreased it with cellulose, and had no effect with inulin or psyllium.

Table 4. Immune parameters in the blood plasma of rats fed experimental diets (n = 10 per group)

Indices	IgM, µg/ml	IgG, µg/ml	IgA, µg/ml	IL-2, ng/l	IL-6, ng/ml	TNFα, pg/ml	CRP, ng/ml	ALB, µmol/l
Control C	936	4085	6190	253	62.7	733	18.3	459
Control CH	875	4208	6277	435	59.6	696	17.1	460
2-way ANOVA:								
CN	860^{a}	4239^{a}	5516$^{a\#}$	334$^{bc\#}$	59.1	663^{a}	22.7$^{a\#}$	466
CNH	734^{b}	4379^{a}	5806^{a}	431^{a}	59.3	650^{ab}	14.3$^{cd\&}$	464
PN	888^{a}	4329^{a}	5715^{a}	368$^{b\#}$	60.7	656^{a}	10.4$^{d\#}$	459
PNH	665$^{b\&}$	3709$^{b\&}$	3764$^{bc\&}$	312$^{c\&}$	58.6	517$^{c\&}$	18.5^{bc}	451
JN	687$^{b\#}$	4090^{ab}	4240$^{b\#}$	264^{d}	52.2$^{\#}$	548$^{c\#}$	18.9^{ab}	455
JNH	707$^{b\&}$	4077^{ab}	3380$^{c\&}$	241$^{d\&}$	54.8	587$^{abc\&}$	17.3^{bc}	471
SN	624$^{b\#}$	3971^{ab}	3766$^{bc\#}$	262^{d}	51.9$^{\#}$	522$^{c\#}$	17.1^{bc}	459
SNH	675$^{b\&}$	3963^{ab}	4237$^{b\&}$	328$^{bc\&}$	52.8	572$^{bc\&}$	18.3^{bc}	445
SEM	16.190	42.448	126.20	8.568	0.753	10.812	0.509	2.243
Cu-NP dose (D)								
L (6.5 mg/kg)	765	4157	4809	307	56.0	597	17.3	460
H (13 mg/kg)	695	4032	4297	328	56.4	581	17.1	458
P-value	0.028	0.196	0.003	0.037	0.815	0.417	0.823	0.754
Fibre type (F)								
C (cellulose)	797	4309	5661	382	59.2^{a}	656	18.5	465
P (pectin)	777	4019	4740	340	59.7^{a}	587	14.4	455
J (inulin)	697	4083	3810	252	53.5^{b}	568	18.1	463
S (psyllium)	650	3967	4002	295	52.4^{b}	547	17.7	452
P-value	0.003	0.068	<0.001	<0.001	0.001	0.001	0.017	0.227
Interaction D×F								
P-value	0.008	0.032	<0.001	<0.001	0.757	0.004	<0.001	0.178

dietary treatments used in the experimental feeding period: groups C and CH – fed a control diet with tested and enhanced Cu content in the mineral mixture (6.5 and 13 mg/kg from $CuCO_3$, respectively) with 8% of cellulose as dietary fibre source; groups CN and CNH – fed diets with supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 8% of cellulose dietary fibre source; groups PN and PNH – fed diets with supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of pectin dietary fibre source; groups JN and JNH – fed diets with supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of inulin dietary fibre source; groups SN and SNH – fed diets with supplementation of CuNPs (6.5 and 13 mg/kg from Cu-nanoparticles, respectively) with 2% of cellulose and 6% of psyllium dietary fibre source; L – treatment (n = 40) with dietary CuNPs 6.5 mg/kg dose; H – treatment (n = 40) with dietary CuNPs 13 mg/kg dose; Ct – reatment (n = 20) with cellulose as dietary fibre; P – treatment (n = 20) with pectin as dietary fibre; J – treatment (n = 20) with inulin as dietary fibre; S – treatment (n = 20) with psyllium as dietary

Discussion

Replacing cellulose with fermentable fibres, such as inulin or pectin, attenuated inflammation, as reflected by lower white blood cell (WBC) and lymphocyte (LYM) counts, and reduced serum interleukin-6 (IL-6) concentrations. This effect is likely associated with microbial fermentation of these fibres to short-chain fatty acids (SCFAs), mainly butyrate, acetate, and propionate. This finding is consistent with our previous studies showing that inulin effectively stimulates butyrate and propionate production, whereas pectin enhances colonic bacterial enzyme activity and maintains beneficial microbial metabolic functions (Juśkiewicz et al., 2024). Cu homeostasis is regulated by specific cellular transporters, including divalent metal transporter 1 (DMT1) and Cu transporter 1 (CTR1), which mediate Cu absorption primarily in the duodenum (Turnlund, 1998; Harris, 2001). The efficiency of this process depends on the chemical form and speciation of dietary Cu (Bost et al., 2016). Importantly, the bioavailability and absorption of Cu in the gastrointestinal tract are determined by pH-controlled speciation and solubility. While Cu carbonate ($CuCO_3$) has low solubility at neutral pH, it undergoes rapid dissolution in acidic gastric conditions (pH 1.5–2.0), forming bioavailable Cu^{2+} ions. In the proximal small intestine, these ions precipitate as poorly soluble $Cu(OH)_2$, which results in limited and physiologically regulated absorption (Wu et al., 2021). CuNPs display significantly higher systemic bioavailability compared to $CuCO_3$. Under identical experimental conditions, Cu utilisation (retention) reached 24.6% for CuNPs versus 8.88% for $CuCO_3$ ($P < 0.001$) (Cholewińska et al., 2018).

This difference arises from rapid CuNPs dissolution in acidic gastric juice, with more than 84% Cu release at pH 1.5 within 24 h (Lee et al., 2016), and from dynamic *in vivo* oxidation and transformation of metallic Cu^0 to Cu_2O and CuO, accompanied by the release of bioavailable Cu^{2+} and Cu^+ ions (Karlsson et al., 2008). Dietary amino acids further increase CuNPs dissolution and bioaccumulation, even under neutral intestinal pH conditions (Boyle et al., 2020), which indicates that Cu nanoparticles remain chemically reactive in relevant physiological environments. Consequently, the effective bioavailable dose of CuNPs is approximately twofold higher than the nominal dietary dose relative to $CuCO_3$ (Cholewińska et al., 2018). This difference should be considered when interpreting dose-response relationships and the immunological effects observed in the present study. SCFAs can bind to receptors on immune cell surfaces, inhibiting histone deacetylase activity and NF-κB signalling, thereby downregulating transcription of TNF-α, IL-6, and CRP genes. They also affect the maturation and function of antigen-presenting cells and macrophages, inducing changes in their phenotype towards an anti-inflammatory profile and supporting the development and activation of regulatory T cells (Tregs), which suppress excessive immune responses (Foey, 2011; Kim et al., 2013; Beukema et al., 2020; Kim, 2023; Sheng et al., 2023). Pectin may exert additional immunomodulatory effects through interaction with pattern recognition receptors, which inhibits LPS-induced IL-6 release from macrophages. Inulin, in turn, suppresses NF-κB pathway activity in epithelial cells and macrophages, directly reducing the production of IL-6 and other pro-inflammatory cytokines. Moreover, both fibres strengthen intestinal barrier function, as demonstrated in our earlier work (Cholewińska et al., 2023). The literature indicates that this effect occurs mainly through SCFA-mediated stimulation of epithelial cell proliferation and increased expression of tight junction proteins, which limits endotoxin translocation and secondary leukocyte activation (Blanco-Pérez et al., 2021; Li et al., 2023; Sheng et al., 2023). Our previous studies also showed that inulin supports DNA repair mechanisms in small-intestinal epithelial cells, while pectin inhibits inflammatory processes that induce apoptosis in these cells (Cholewińska et al., 2023). Additionally, pectin, through its galacturonic acid residues, can chelate metal ions, and thus reduce Cu-induced ROS formation (Lara-Espinoza et al., 2018). These complex molecular mechanisms may explain the haematological and biochemical changes occurring independently of Cu dose and form, showing the universal anti-inflammatory properties of fermentable fibres.

Partial replacement of cellulose with psyllium husk, in combination with CuNPs at the tested Cu exposure dose, increased white blood cell and lymphocyte counts, in contrast to the anti-inflammatory effects observed for inulin and pectin. This difference likely reflects the distinct physicochemical and biological properties of psyllium. Unlike highly fermentable fibres, psyllium is only partially fermented and acts mainly through gel formation and bile acid (BA) sequestration, which increases circulating BA levels and activates the farnesoid X receptor (FXR). Activation of the latter receptor may exert immunostimulatory effects in certain conditions by promoting leukopoiesis and lymphocyte proliferation. In addition, limited fermentation of psyllium results in lower SCFA production compared to inulin or pectin, which reduces SCFA-mediated anti-inflammatory signalling through inhibition of the NF-κB pathway. Psyllium may also alter the gut microbiota composition in a manner that transiently increases immune cell production. Elevated BA flow can induce stress responses that mobilise WBC (Bretin et al., 2023). Moreover, although the gelling viscosity of psyllium may slow intestinal passage, it does not chelate Cu ions as effectively as the charged groups present in pectin. Prolonged transit may therefore extend mucosal exposure to CuNPs and lead to mild immune activation. In contrast, Bretin et al. (2023) reported that psyllium-enriched diets protected against colonic inflammation through mechanisms independent of SCFA or IL-22 signalling and requiring only limited microbiota involvement. In human studies, psyllium has shown inconsistent effects on inflammatory markers, with some trials reporting no changes in CRP or IL-6 concentrations. These findings indicate that its impact on immune parameters may vary with Cu dose or form (King et al., 2008).

The results of the present study demonstrated that partial replacement of cellulose with inulin or psyllium husk in a diet containing CuNPs at the experimental Cu exposure dose (6.5 mg Cu/kg) reduced plasma levels of IgM, IL-6, and TNF-α, which indicates attenuation of both humoral and innate inflammatory responses. Moreover, supplementation with pectin, inulin, or psyllium husk, combined with a two fold higher CuNPs dose (13 mg Cu/kg), intensified this effect and additionally reduced IgA and IL-2 levels. It can be hypothesised that the increased production of butyrate and propionate during fermentation of inulin and pectin inhibited histone deacetylases, thereby limiting immunoglobulin

class-switch recombination and consequently decreasing IgA and/or IgM secretion (Foey, 2011). This mechanism of restricted class switching may also account for the reduced plasma IgG levels observed in rats fed pectin together with the high CuNPs dose. Concurrently, SCFA signalling may inhibit NFκB and AP-1 activity in T lymphocytes, downregulating IL-2 transcription and helper T cell cytokine production (Foey, 2011). Psyllium may further contribute to this effect by forming viscous gels that delay antigen absorption and reduce interactions between pathogen-associated molecular patterns (PAMPs) and Toll-like receptors (TLRs) on immune cells, further reducing TNF-α release (Bretin et al., 2023). It is also plausible that increased uptake of CuNPs at the higher dietary dose directly affects dendritic cell maturation and cytokine profiles, promoting regulatory immune phenotypes and reinforcing anti-inflammatory signals derived from dietary fibres (Dürholz et al., 2020).

Replacing inorganic Cu with CuNPs at the experimental dose in rats fed a cellulose-only fibre diet decreased plasma IgA concentrations and was accompanied by elevated C-reactive protein (CRP) levels.

It should be noted that metallic CuNPs administered in the diet may undergo partial oxidation and dissolution under physiological gastrointestinal conditions, resulting in the release of Cu^{2+} ions, as well as the formation of secondary agglomerates. Numerous studies have demonstrated that CuNPs are not chemically inert *in vivo* and may undergo dynamic transformations depending on pH, redox potential, and the presence of biological ligands, such as bile salts, digestive enzymes, and microbial metabolites. Consequently, the observed immunological and inflammatory effects likely reflect the combined action of nanoparticulate Cu and released ionic Cu rather than a response to intact metallic nanoparticles alone. This dual exposure may potentiate oxidative stress and inflammatory signalling, particularly in the absence of fermentable fibre-derived SCFAs that can counteract Cu-induced ROS generation (Karlsson et al., 2008; Studer at al., 2010). The decrease in plasma IgA concentrations suggests that the increased bioavailability of CuNPs may directly impair mucosal B-cell function, possibly through oxidative stress and apoptosis of lamina propria plasma cells. This interpretation is consistent with murine inhalation models, in which CuO nanoparticles activated immune cells while reducing the viability of antibody-secreting cells (Tulinska et al., 2022). The concomitant rise in CRP level reflects an acute-phase response resulting from hepatic synthesis in reaction to nanoparticle-induced systemic inflammation and ROS generation. Similar effects were reported in spontaneously hypertensive rats exposed to CuO nanoparticles, which showed increased serum CRP levels (Wang et al., 2022). These divergent responses, i.e., IgA suppression together with CRP elevation, indicate dual effects of CuNPs. Dietary CuNPs may increase ROS formation, which activates hepatic NF-κB and stimulates CRP synthesis, while simultaneously inducing endoplasmic reticulum stress in B cells and restricting immunoglobulin class switching to IgA. This mechanism links a systemic acute-phase response with local immunosuppression. Interestingly, rats receiving cellulose with the high CuNPs dose displayed reduced plasma CRP levels, which may be explained by oxidative hormesis and changes in hepatic redox signalling pathways. In the absence of fermentable SCFA precursors, elevated nanoparticle concentrations may induce moderate oxidative stress that activates the Nrf2 pathway in hepatocytes. This activation stimulates the expression of antioxidant enzyme genes (e.g., HO-1, NQO1) and concurrently suppresses NF-κB-dependent acute-phase protein synthesis, including CRP, as an adaptive anti-inflammatory response (Al-Ruwad et al., 2024). Moreover, under low intestinal fermentation conditions, reduced proinflammatory cytokine signalling, especially IL-6-mediated hepatocyte stimulation, may further inhibit CRP production despite increased CuNPs bioaccumulation (Sutunkova et al., 2023).

The dietary combination of psyllium husk with a high CuNPs dose also altered erythrocyte and platelet indices, indicative of mild haemolytic anaemia and reactive thrombopoiesis. Mean corpuscular haemoglobin (MCH) decreased, suggesting reduced haemoglobin concentration per erythrocyte, likely due to impaired haemoglobin synthesis associated with micronutrient imbalance and oxidative damage (Tesser et al., 2020). At the same time, higher red cell distribution width (RDWc) reflects elevated anisocytosis caused by premature release of erythrocytes into the circulation to compensate for accelerated removal of damaged cells. Similar phenomena have been observed in rodent models exposed to CuNPs, in which oxidative membrane damage accelerated erythrocyte fragmentation (Karlsson et al., 2013). An increase in mean platelet volume (MPV) further confirms reactive thrombocytosis associated with systemic inflammatory and oxidative stimuli. Platelet progenitor cells produce larger and more reactive platelets under stress to maintain

haemostasis during intravascular haemolysis. The gel-forming matrix of psyllium husk may exacerbate these effects by altering Cu absorption kinetics, causing local fluctuations in Cu availability that impair red blood cell maturation and increase CuNPs-induced ROS generation.

Although the present findings provide valuable information on the interactions between dietary fibre types and CuNPs in relation to immune function, several limitations should be noted. First, the rodent model, despite its wide use, does not fully reproduce human gastrointestinal physiology or microbiome diversity, which could potentially limit the direct extrapolation of these results to humans. Second, the CuNPs used in this study (40 nm) represent only one nanoparticle formulation. Biological effects may differ substantially depending on particle size and surface characteristics. Future research should investigate doseresponse relationships over a broader range of CuNPs concentrations and particle sizes, as well as the long-term effects of their interaction with various dietary fibres on the immunological status of experimental animals. Additionally, detailed mechanistic studies are required to elucidate the molecular pathways responsible for the modulatory properties of different fibre types in the presence of CuNPs. Such research will significantly advance our understanding of these complex interactions and facilitate the development of targeted nutritional strategies.

Conclusions

During supplementation with the elevated copper(II) carbonate ($CuCO_3$) dose, pectin exerted the most beneficial effects on immunological parameters and demonstrated stronger anti-inflammatory activity compared to inulin or psyllium husk. Replacement of the traditional copper source with nanoparticles, even at the lower experimental dose, exacerbated inflammation and impaired immune function in rats. Partial substitution of cellulose with inulin or pectin in the rats' diet mitigated the adverse effects induced by copper nanoparticles (CuNPs) regardless of dose, with inulin showing the strongest anti-inflammatory effect of the alternative dietary fibres.

Conflict of interest

The Authors declare that there is no conflict of interest.

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Oświadczenia doktoranta oraz współautorów dotyczących ich wkładu w przygotowanie publikowanych prac naukowych

Załącznik nr 7 – Oświadczenie o współautorstwie

Lublin, 02.02.2026

lek. wet. Aleksandra Marzec
ul. Akademicka 13
Lublin, 20-950

Rada Dyscypliny Zootechnika i Rybactwo
Uniwersytetu Przyrodniczegow
Lublinie

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy:

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Aleksandra Marzec

Podpis

Załącznik nr 7 – Oświadczenie o współautorstwie

Lublin, 02.02.2026

dr Ewelina Cholewińska
ul. Akademicka 13
Lublin, 20-930

Rada Dyscypliny Zootechnika i Rybactwo
Uniwersytetu Przyrodniczego
w Lublinie

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Ewelina Cholewińska

Podpis

Załącznik nr 7 – Oświadczenie o współautorstwie

Lublin, 02.02.2026

Dr hab. Anna Stępniowska
ul. Akademicka 13
Lublin, 20-930

Rada Dyscypliny Zootechnika i Rybactwo
Uniwersytetu
Przyrodniczegow Lublinie

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Anna Stępniowska

Podpis

Załącznik nr 7 – Oświadczenie o współautorstwie

Lublin, 02.02.2026

Prof. dr hab. Katarzyna Ognik
ul. Akademicka 13
Lublin, 20-930

Rada Dyscypliny Zootechnika i Rybactwo
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Przyrodniczego w Lublinie

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Katarzyna Ognik
..... Podpis

Załącznik nr 7 – Oświadczenie o współautorstwie

Lublin, 02.02.2026

dr hab. n. med. Wojciech
Dworzański

Rada Dyscypliny Zootechnika i Rybactwo
Uniwersytetu Przyrodniczegow
Lublinie

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Mój udział polegał na pomocy w interpretacji wyników i zatwierdzeniu ostatecznej wersji publikacji.

Dworzański

Podpis

Załącznik nr 7 – Oświadczenie o współautorstwie

Lublin, 02.02.2026

dr hab. n.wet., dr n. pr. Piotr Listos,
prof. uczelni
ul. Głęboka 30E
Lublin, 20-400

Rada Dyscypliny Zootechnika i Rybactwo
Uniwersytetu Przyrodniczegow
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Lublin, 02.02.2026

dr hab. Bartosz Fotschki
ul. Trylińskiego 18
Olsztyn, 10-683

Rada Dyscypliny Zootechnika i Rybactwo
Uniwersytetu Przyrodniczego
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Bartosz Fotschki
Podpis

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Lublin, 02.02.2026

dr Joanna Fotschki
ul. Trylińskiego 18
Olsztyn, 10-683

Rada Dyscypliny Zootechnika i Rybactwo
Uniwersytetu Przyrodniczegow
Lublinie

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Joanna Fotschki
Podpis

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Lublin, 02.02.2026

prof. dr hab. inż. Jerzy Juśkiewicz
ul. Trylińskiego 18
Olsztyn, 10-683

Rada Dyscypliny Zootechnika i Rybactwo
Uniwersytetu Przyrodniczegow
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mgr inż. Dorota Napiórkowska
ul. Trylińskiego 18
Olsztyn, 10-683

Rada Dyscypliny Zootechnika i Rybactwo
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Napiórkowska

Podpis

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dr Przemysław Sołek
ul. Akademicka 13
Lublin, 20-930

Rada Dyscypliny Zootechnika i Rybactwo
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