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ROZPRAWA DOKTORSKA

Ocena wpływu niskiej i wysokiej dawki mikroplastiku na kodowanie chemiczne jelitowego układu nerwowego i układ immunologiczny jelita cienkiego świni domowej

Evaluation of the effects of low and high doses of microplastic on the chemical coding of the enteric nervous system and the immune system of the small intestine in domestic pigs

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1. Streszczenie

Mikroplastik to drobne cząsteczki tworzyw sztucznych charakteryzujące się nieregularnym kształtem i średnicą między 0,1 a 5000 μm. Powszechne używanie tworzyw sztucznych w praktycznie każdej sferze życia człowieka powoduje, że występuje on w wodzie, glebie czy powietrzu, na każdym obszarze kuli ziemskiej. Jest obecny w paszy dla zwierząt, napojach butelkowanych (wodzie pitnej, piwie), soli, warzywach i owocach, ale również produktach odzwierzęcych (mięsie, miodzie). Politereftalan etylenu (PET) jest tworzywem wykorzystywanym w łańcuchu żywnościowym do pakowania żywności. Przewód pokarmowy z kolei stanowi jedną z pierwszych barier obronnych przeciwko mikroplastikowi, który może indukować jego stan zapalny. Prawidłowe funkcjonowanie przewodu pokarmowego możliwe jest dzięki m. in. jelitowemu układowi nerwowemu. Reguluje on aktywność wydzielniczą, przepływ krwi i motorykę. Syntetyzowane i wydzielane przez niego substancji biologicznie czynne uczestniczą w neuroimmunoregulacji. Proces ten pod wpływem różnych czynników patologicznych czy ksenobiotyków jak np. mikroplastik może ulegać zmianom.

W celu określenia czy i jak mikroplastik PET w dawce 0,1 g/dzień i 1 g/dzień wpływa na funkcję jelitowego układu nerwowego jelita cienkiego świni, wybrano sześć substancji biologicznie czynnych (transkrypt regulowany przez kokainę i amfetaminę, galaninę, neuronalną formę syntazy tlenku azotu, substancję P, pęcherzykowy transporter acetylocholiny i wazoaktywny peptyd jelitowy) i określono odsetek neuronów immunoreaktywnych względem tych substancji przy użyciu barwień immunofluorescencyjnych. Zbadano również stężenie pięciu wybranych cytokin (interleukina 1β, interleukina 6, interleukina 8, interleukina 10 oraz czynnik martwicy nowotworów α) w ścianie jelita krętego z wykorzystaniem testów ELISA.

Wpływ dawki 0,1 g/dzień był różny (brak zmian, zwiększenie lub zmniejszenie odsetka neuronów immunoreaktywnych) w zależności od badanego odcinka jelita cienkiego oraz splotu jelitowego układu nerwowego. Dawka 1 g/dzień nie zmieniła jedynie odsetka populacji neuronów CART-dodatnich (jelito kręte, splot mięśniowy) i nNOS-dodatnich (jelito czcze, splot podśluzowy zewnętrzny). W pozostałych przypadkach odnotowano statystycznie istotne zmiany polegające na zwiększeniu lub zmniejszeniu odsetka neuronów immunoreaktywnych wobec badanych substancji.

Analiza stężenia cytokin w ścianie jelita krętego wykazała, że zarówno dawka mikroplastiku 0,1 g/dzień oraz 1 g/dzień nie zmienia ich koncentracji w sposób istotny statystycznie.

Uzyskane wyniki sugerują, że mikroplastik wpływa na kodowanie chemiczne neuronów jelitowego układu nerwowego. Oddziaływanie mikroplastiku na przewód pokarmowy jest zależne od zastosowanej dawki oraz badanego odcinka jelita cienkiego. Powstałe różnice mogą wynikać ze zmian w właściwościach fizykochemicznych cząsteczek powstałych pod wpływem pasażu z treścią pokarmową przez przewód pokarmowy i być efektem ich oddziaływania mechanicznego i/lub chemicznego.

W związku z powszechnością mikroplastiku wskazane są dalsze badania mające na celu określenie wpływu mikroplastiku o odmiennych właściwościach (wielkość, kształt, rodzaj polimeru, pierwotny lub wtórny) na organizmy ludzi i zwierząt.

Słowa kluczowe: mikroplastik, *Sus scrofa domesticus*, jelitowy układ nerwowy, cytokiny, neuroprzekaźniki, PET

2. Abstract

Microplastics are small plastic particles characterized by irregular shapes and diameters ranging from 0,1 to 5000 μm. Their widespread use across virtually every aspect of human life results in their presence in water, soil, and air across all regions of the globe. Microplastics have been detected in animal feed, bottled beverages (including drinking water and beer), salt, vegetables, fruits, and even animal-derived products (meat, honey). Polyethylene terephthalate (PET) is a plastic commonly used in the food industry, particularly for food packaging. The gastrointestinal tract serves as one of the primary barriers against microplastics, which may induce inflammation. Its proper function is supported, among other factors, by the enteric nervous system, which regulates secretion, vasodilation, and motility. The biologically active substances synthesized and secreted by it participate in neuroimmunoregulation. It may be altered by pathological factors or xenobiotics, such as microplastics.

To determine whether PET microplastics at doses of 0,1 g/day and 1 g/day affect the function of the enteric nervous system of the small intestine, six biologically active substances (cocaine and amphetamine-regulated transcript, galanin, neuronal nitric oxide synthase, substance P, vesicular acetylcholine transporter, and vasoactive intestinal peptide) were selected. The percentage of neurons immunoreactive to these markers was analyzed using immunofluorescent staining. Additionally, the concentrations of five selected cytokines (interleukin 1β, interleukin 6, interleukin 8, interleukin 10, and tumor necrosis factor α) in the ileal wall were measured using ELISA test.

The effects of the 0,1 g/day dose varied depending on the section of the small intestine and the enteric plexus examined. Reductions, increases, or no statistically significant differences were noted for this dose.

A dose of 1 g/day did not alter the population of CART-positive neurons (ileum, myenteric plexus) or nNOS-positive neurons (jejunum, outer submucosal plexus). In other cases, statistically significant changes (increases or decreases) were observed.

The effect of the dose of 0.1 g/day was different (no change, increase or decrease in the percentage of immunoreactive neurons) depending on the examined section of the small intestine and enteric plexus of the nervous system.

The results indicate that neither dose caused a statistically significant increase or decrease in the concentrations of the selected cytokines in the ileal wall.

The results confirm that microplastics affect the chemical coding of neurons of the enteric nervous system. The impact of microplastics depends on the administered dose

and the specific section of the small intestine studied. The observed differences may result from changes in the physicochemical properties of the particles caused by their passage with digestive contents through the gastrointestinal tract and may be the result of the mechanical and/or chemical impact of microplastics.

Due to the ubiquity of microplastics, further research is recommended to evaluate the effects of microplastics with different properties (size, shape, type of polymer, primary or secondary) on the bodies of humans and animals.

Keywords: microplastic, *Sus scrofa domesticus*, enteric nervous system, cytokines, neurotransmitters, PET

3. Wykaz skrótów

ACh – acetylocholina

AChE – acetylocholinoesteraza

C – grupa kontrolna

CART – transkrypt regulowany przez kokainę i amfetaminę

EFSA – Europejski Urząd ds. Bezpieczeństwa Żywności

ENS – jelitowy układ nerwowy (ang. enteric nervous system)

GAL – galanina

GALT – tkanka limfatyczna związana z jelitami

HD – grupa otrzymująca wysoką dawkę MP-PET (1 g/dzień)

IL-10 – interleukina10

IL-1β – interleukina 1 beta

IL-6 – interleukina 6

IL-8 – interleukina 8

ISP – splot podśluzowy wewnętrzny (ang. inner submucous plexus)

LD – grupa otrzymująca niską dawkę MP-PET (0,1 g/dzień)

MP – splot mięśniowy (ang. myenteric plexus)

MP-PET – mikroplastik politereftalanu etylenu

nNOS – izoforma neuronalna syntazy tlenku azotu

NO – tlenek azotu

OSP – splot podśluzowy zewnętrzny (ang. outer submucous plexus)

PET – politereftalan etylenu

PGP 9.5 – produkt genu proteinowego 9.5

PS - polistyren

SP – substancja P

TNF-α – czynnik martwicy nowotworów alfa

VAChT – pęcherzykowy transporter acetylocholiny

VIP – wazoaktywny peptyd jelitowy

4. Wstęp

4.1. Mikroplastik

Mikroplastik to drobne cząsteczki tworzyw sztucznych, które według Europejskiego Urzędu ds. Bezpieczeństwa Żywności (EFSA) charakteryzują się nieregularnym kształtem i średnicą między 0,1 a 5000 µm (EFSA Panel on Contaminants in the Food Chain, 2016). Jest to najszerzej rozpowszechniona definicja. Z biegiem czasu i intensyfikacją badań nad mikroplastikiem wysnuto postulaty mające na celu doprecyzowanie definicji mikrocząsteczek. W przypadku klasyfikacji badacze są zgodni pod kątem źródła mikroplastiku (rodzaj polimeru oraz rozróżnienie na mikroplastik pierwotny - wyprodukowany w danej wielkości i kształcie i wtórny – powstały pod wpływem degradacji), kształtu cząsteczek (sferyczne, włókna, nieregularne) i koloru (bezbarwne, kolorowe) (Kadac-Czapska et al., 2023). Największe wyzwanie stanowi jednak wielkość cząsteczek. Aktualnie można wyróżnić mega-, makro-, mezo-, mikro- i nanoplastik (Corte Pause et al., 2024). Jednak w zależności od źródła, przedziały wielkości cząsteczek różnią się. Może to powodować późniejsze nieporozumienia w dyskusji na temat mikroplastiku i wskazuje na potrzebę ujednolicenia tej definicji. Z tego względu w niniejszej dysertacji przyjęto definicję zaproponowaną przez EFSA.

Wszechobecność mikroplastiku jest bezdyskusyjna. Za główne drogi narażenia przyjmuje się drogę *per os* oraz wziewną (Li et al., 2023). Literatura również podaje drogę przezskórną, jednak ma ona mniejsze znaczenie (Liu & You, 2023). Masowe używanie tworzyw sztucznych w praktycznie każdej sferze życia człowieka powoduje, że występuje on w wodzie, glebie czy powietrzu, na każdym obszarze kuli ziemskiej. Jest on obecny w paszy dla zwierząt, napojach butelkowanych (np. wodzie pitnej, piwie), soli, warzywach i owocach, ale również produktach odzwierzęcych (np. mięsie, miodzie) (Akanyange et al., 2022; Cox et al., 2019; Zhang et al., 2020). Ponieważ tworzywa sztuczne są szeroko stosowane w sektorze rolno-spożywczym, łańcuch żywnościowy jest krytycznym punktem ekspozycji konsumentów na mikrocząsteczki. Z punktu widzenia europejskich inicjatyw takich jak Farm to Fork („od pola do stołu”) obecność mikroplastiku w łańcuchu żywnościowym zagraża zdrowiu i bezpieczeństwu konsumentów (Corte Pause et al., 2024; Kadac-Czapska et al., 2023; Siddiqui et al., 2023), jednak wyeliminowanie zagrożenia związanego z mikroplastikiem wydaje się aktualnie nierealne. Mikroplastik odgrywa kluczową rolę w inicjatywie "One Health" („Jedno Zdrowie”), która integruje zdrowie ludzi, zwierząt i środowiska, podkreślając ich wzajemne powiązania.

Gdy frazę „mikroplastik" wprowadzi się do baz danych PubMed, Web of Science lub Scopus, można zaobserwować rosnący z roku na rok trend w ilości publikacji naukowych na ten temat. Zakres badań przedstawionych w literaturze jest szeroki. Dominują badania wpływu mikroplastiku na zwierzęta takie jak skorupiaki, ryby lub gryzonie przy zastosowaniu metod *in vivo* (Liu & You, 2023; Prüst et al., 2020). Dostępne są także pojedyncze badania wykorzystujące kurczęta (Chen et al., 2023). Istnieją też liczne doniesienia z wykorzystaniem różnych modeli *in vitro* (Liu & You, 2023; Stock et al., 2019). Badania oparte na świńskim modelu badawczym skupiają się głównie na identyfikacji mikroplastiku w odchodach tych zwierząt (Yang et al., 2021). Przeprowadzone przez nas doświadczenie jest jednym z pierwszych wykorzystującym świnie domową (*Sus scrofa domesticus*) jako model badawczy w warunkach *in vivo*.

U zwierząt pod wpływem ekspozycji na mikroplastik obserwowano m. in. zmniejszenie przyrostów masy ciała, pogorszenie jakości mięsa, dysbiozę, zaburzenia w układzie rozrodczym czy nawet śmierć (Chen et al., 2023; Ghosh et al., 2021; Yang et al., 2024; Zou et al., 2024). U brojlerów wykazano, że polistyren (PS) przyczynił się do zwiększenia masy mięśniowej, co jest zjawiskiem pożądanym, ale jakość mięsa uległa pogorszeniu (Chen et al., 2023). Określenie wpływu zanieczyszczenia mikroplastikiem jest istotne, ponieważ może on wpływać na chów i hodowlę zwierząt, których tkanki (w tym mięso), wykorzystywane są przez ludzi w łańcuchu żywnościowym.

Należy zaznaczyć, że dominujący rodzaj tworzywa sztucznego wykorzystywanego w badaniach to PS. Dopiero w niedawnym czasie w procedurach eksperymentalnych zaczęto szerzej wykorzystywać inne rodzaje tworzyw jak np. politereftalan etylenu (PET). Rodzaj zastosowanych cząsteczek potencjalnie może mieć wpływ na uzyskane wyniki. Związane jest to choćby z właściwościami fizykochemicznymi danego tworzywa.

Opakowania plastikowe wykonane z PET są szeroko stosowane w przemyśle spożywczym ze względu na ich zalety takie jak wysoka wytrzymałość, trwałość i niska masa. Szacuje się, że z 0,5-litrowej butelki PET może powstać do 480 milionów cząsteczek, które następnie mogą być uwalniane do środowiska (Pinlova & Nowack, 2024). Samo otwieranie butelek PET, zwłaszcza wielokrotne, może doprowadzić do uwolnienia cząsteczek do wody (Winkler et al., 2019). Zastosowanie PET w prezentowanych doświadczeniach wypełnia więc, istniejącą lukę badawczą.

Przewód pokarmowy odgrywa kluczową rolę w obronie organizmu przed ksenobiotykami, w tym przeciwko mikroplastikowi. Jego cząsteczki mogą zostać połknięte wraz z pożywieniem lub napojami. Aktualny stan wiedzy pozwala na stwierdzenie, że wraz

ze zmniejszeniem wielkości cząsteczek wzrasta możliwość wywołania negatywnych efektów przez mikroplastik (Li et al., 2023). Cząsteczki dostatecznie małe <150 µm mogą przekraczać barierę błony śluzowej jelit, a <1,5 µm mogą być transportowane do innych narządów (EFSA Panel on Contaminants in the Food Chain, 2016; Stock et al., 2019). Stąd też doniesienia o obecności mikroplastiku m. in. w jądrach, wątrobie, ale również w mleku, plwocinie, krwi czy łożysku (Barceló et al., 2023).

Mechanizm oddziaływania mikroplastiku na organizmy ludzi i zwierząt nie został jeszcze w pełni poznany. Aktualne dane wskazują, że opiera się on na wywoływaniu stresu oksydacyjnego, powodowaniu mechanicznych uszkodzeń komórek i tkanek, genotoksyczności, zaburzeniach w funkcjonowaniu białek, lipidów, DNA oraz neurotoksyczności (Li et al., 2023; Płuciennik et al., 2024).

4.2. Jelitowy układ nerwowy

Jelitowy układ nerwowy (ang. enteric nervous system, ENS), będący częścią autonomicznego układu nerwowego, reguluje jego pracę poprzez kontrolowanie mięśniówki, naczyń krwionośnych czy aktywność wydzielniczą nabłonka błony śluzowej (Furness, 2008). Ze względu na ilość neuronów jego budowa różni się w zależności od gatunku zwierzęcia. W związku z dużym podobieństwem pomiędzy świnią domową a człowiekiem, zwłaszcza w budowie anatomicznej i fizjologii przewodu pokarmowego, zwierzęta te są często wykorzystywane w badaniach biomedycznych, w tym właśnie nad wspomnianym układem (Lunney et al., 2021). U ludzi i u świń ENS zbudowany jest ze splotu mięśniowego (ang. myenteric plexus, MP) oraz splotu podśluzowego podzielonego na zewnętrzny (ang. outer submucous plexus, OSP) i wewnętrzny (ang. inner submucous plexus, ISP) splot podśluzowy (Szymańska & Gonkowski, 2018). Poprzez wydzielanie substancji neuronalnie czynnych w wymienionych splotach, ENS może regulować pracę przewodu pokarmowego w odpowiedzi na aktualne warunki mikrośrodowiska jelitowego (Furness, 2008).

Jelitowy układ nerwowy charakteryzuje się dużymi zdolnościami adaptacyjnymi. Pod wpływem różnych czynników patologicznych na jego terenie może dochodzić do zmian kodowania chemicznego neuronów, co jest odpowiedzią organizmu mającą na celu dążenie do homeostazy. Również niektóre choroby neurodegeneracyjne mogą powodować zaburzenia w funkcjonowaniu ENS (Chalazonitis & Rao, 2018; De Giorgio & Camilleri, 2004; Holland et al., 2021).

W celu określenia czy mikroplastik PET w dawce 0,1 g/dzień i 1 g/dzień wpływa na funkcję ENS wybrano sześć substancji neuronalnie aktywnych. Dokonano analizy zmienności ilościowej neuronów ENS immunoreaktywnych wobec tych substancji, wykorzystując technikę immunocytochemiczną.

Transkrypt kodowany amfetaminą i kokainą (CART) jest peptydem, którego funkcja w przewodzie pokarmowym nie została jeszcze w pełni poznana. Aktualny stan wiedzy wskazuje, że CART może pełnić funkcje neuroprotekcyjne, regulujące motorykę (hamuje opróżnianie żołądka) i hamujące wydzielanie soku żołądkowego (Okumura et al., 2000).

Galanina (GAL) jest neuropeptydem szeroko rozpowszechnionym w przewodzie pokarmowym. Hamuje ona motorykę jelit i opróżnianie żołądka, uczestniczy w procesach zapalnych (wykazuje działanie neuroprotekcyjne, pośredniczy i uczestniczy w interakcjach między układem odpornościowym i nerwowym oraz ma działanie antyproliferacyjne i proapoptotyczne), reguluje aktywność wydzielniczą (hamuje wydzielanie somatostatyny, insuliny, glukozy i kwasu żołądkowego) (Brzozowska & Całka, 2021).

Tlenek azotu (NO) syntetyzowany jest przez syntazę tlenku azotu (NOS), która występuje w trzech izoformach: neuronalnej (nNOS), endotelialnej (eNOS) oraz indukowanej (iNOS). Tlenek azotu może pełnić funkcje neuroprotekcyjne, ale również może powodować uszkodzenie komórek (Calabrese et al., 2007). Jest on odpowiedzialny za regulację motoryki przewodu pokarmowego (działa rozkurczająco) oraz przepływu krwi (Takahashi, 2003). Tlenek azotu zmniejsza wydzielanie kwasu żołądkowego w żołądku, a w dwunastnicy i jelicie grubym pobudza funkcję wydzielniczą (Szymańska et al., 2018).

Substancja P (SP) jest przedstawicielem tachykinin. Jej zwiększona ekspresja powiązana jest z występowaniem stanów zapalnych oraz nocycepcją. Substancja P pobudza prozapalnie limfocyty i makrofagi, zwiększa produkcję prozapalnych cytokin TNF-α, IL-1β, IL-6 i IL-12, wykazuje działanie prowydzielnicze i rozszerzające naczynia krwionośne (Koon & Pothoulakis, 2006)

Neurony cholinergiczne reprezentowane są przez pęcherzykowy transporter acetylocholiny (VAChT). Acetylocholina (ACh) jest jednym z najszerzej rozpowszechnionych neurotransmiterów w przewodzie pokarmowym. Jest transportowana przez VAChT do pęcherzyków synaptycznych i występuje w zakończeniach neuronów cholinergicznych (Harrington et al., 2010). Acetylocholina reguluje motorykę jelit (skurcz mięśni) oraz funkcje wydzielnicze (Hansen, 2003; Harrington et al., 2010). Wykazuje ona również aktywność przeciwzapalną obniżając poziom cytokin prozapalnych (Van Der Zanden et al., 2009).

Wazoaktywny peptyd jelitowy (VIP) jest jednym z głównych neuropeptydów o właściwościach hamujących motorykę jelit. Działa neuroprotekcyjne poprzez utrzymanie równowagi pomiędzy cytokinami pro- i przeciwzapalnymi, wpływa na przeżywalność neuronów, hamuje aktywność (fagocytozę i chemotaksję) makrofagów, stymuluje różnicowanie limfocytów B i produkcję IgA, hamuje proliferacje i migracje limfocytów T oraz wydzielanie cytokin prozapalnych, takich jak TNF-α, IFN-γ, IL-6, IL-12, zwiększa wydzielanie IL-10 (Arciszewski & Ekblad, 2005; Janjatović et al., 2012; Zacharko-Siembida et al., 2013). Acetylocholina i VIP wiążą się z receptorami nabłonkowymi i stymulują wydzielanie płynów i chlorków oraz hamują wchłanianie sodu (Hansen, 2003)

Dotychczas brak w literaturze badań dotyczących wpływu mikroplastiku na ENS. Dostępne są publikacje pokazujące wpływ mikroplastiku na neurony i neurotransmitery w ośrodkowym układzie nerwowym (Prüst et al., 2020). Wykazano m. in., że mikroplastik może powodować neurotoksyczność poprzez uszkodzenia neuronów cholinergicznych, GABAergicznych, dopaminergicznych czy glutaminergicznych oraz, że może prowadzić do zahamowania aktywności acetylocholinoesterazy (AChE) (Sofield et al., 2024).

Neurotoksyczność mikroplastiku może być powiązana z osią jelitowo-mózgową poprzez zaburzenie integralności błony śluzowej jelit, wywołanie dysbiozy, zmniejszone wydzielanie mucyny (Grodzicki et al., 2021; Sofield et al., 2024). W konsekwencji mikroplastik może być potencjalnie jednym z czynników biorących udział w patogenezie chorób neurodegeneracyjnych jak choroba Alzheimera lub choroba Parkinsona (Sofield et al., 2024).

4.3. Tkanka limfatyczna związana z jelitami

Oś jelitowo-mózgowa jest to dwukierunkowa oś regulacyjna pomiędzy tymi narządami (Asgari et al., 2024; Sofield et al., 2024). Jelitowy układ nerwowy, mikrobiom jelitowy oraz tkanka limfatyczna związana z jelitami (GALT) są jej istotnymi częściami (Asgari et al., 2024). Wykazano, że nieprawidłowości w działaniu osi jelitowo-mózgowej mogą prowadzić do szeregu zaburzeń takich jak stany depresyjne, lękowe, schizofrenia, migrena, choroba Huntingtona czy stwardnienie zanikowe boczne (Asgari et al., 2024). Potwierdzono także zależność pomiędzy stanami zapalnymi przewodu pokarmowego, a chorobami ogólnoustrojowymi (Lau et al., 2015; Yamazaki & Kamada, 2023).

Dotychczasowe badania wskazują, że mikroplastik z różnych tworzyw sztucznych i wielkości może wpływać na zwiększenie stężenia cytokin prozapalnych (Pulvirenti et al., 2022; Sofield et al., 2024). Podczas stanów zapalnych jelit o różnorodnej etiologii,

może dochodzić do zmian w stężeniu cytokin (prozapalnych np. IL-6, IL-12, IL-23 i IL-2 lub przeciwzapalnych np. IL-10 i transformującego czynnika wzrostu β (TGF-β). Określenie schematu zmian w stężeniu wybranych cytokin może stanowić marker, który później ułatwi diagnozowanie patologii na terenie jelita cienkiego pod wpływem mikroplastiku i stanowić może również element celowanej terapii (Neurath, 2014).

Czynnik martwicy nowotworów alfa (TNF-α) jest mediatorem stanu zapalnego. Może zwiększać proliferacje komórek oraz wywoływać zmiany w przepuszczalności bariery nabłonkowej jelit. Zwiększone stężenie TNF-α może być obserwowane przy takich enteropatiach jak choroba Leśniowskiego-Crohna (Dosh et al., 2019).

Interleukina 1β (IL-1β) jest interleukiną o plejotropowym działaniu (Neurath, 2014). Wykazano dodatnią korelację pomiędzy poziomem IL-1β, a nasileniem stanu zapalnego błony śluzowej jelit (Al-Sadi et al., 2008). Wraz z TNF-α wpływa na wzrost przepuszczalności jelit (Dosh et al., 2019), co również często jest obserwowane przy ekspozycji na mikroplastik (Liu & You, 2023).

Interleukina 6 (IL-6) jest kolejną cytokiną o pjeotropowym działaniu, może zarówno zwiększać jak i zmniejszać stan zapalny, a obserwowany efekt zależny jest od komórek docelowych (Pawłowska-Kamieniak et al., 2021). Niedobór IL-6 może zmniejszać aktywację gleju w odpowiedzi na uraz tkanki nerwowej, ale z kolei jej nadprodukcja w mózgu prowadzi do zmian neurodegeneracyjnych (Rothaug et al., 2016). Wykazano również, że zwiększona ekspresja IL-6 w jelicie może korelować, ze zwiększoną ekspresją tej interleukiny w mózgu (Wang et al., 2010). Ponadto stężenie IL-6 koresponduje z nasileniem zmian zapalnych w jelicie (Pawłowska-Kamieniak et al., 2021).

Interleukina 8 (IL-8) może być z powodzeniem używana, jako biomarker stanów zapalnych lub nowotworzenia (Shahzad et al., 2010). Zwiększona produkcja TNF-α oraz IL-1β może prowadzić do indukcji stężenia IL-8, która z kolei jest chemoatraktantem neutrofili (Dosh et al., 2019).

Interleukina 10 (IL-10) jest cytokiną o właściwościach przeciwzapalnych. Wydzielana jest przez szeroką gamę komórek i ma plejotropowe działanie na limfocyty T, limfocyty B, komórki mieloidalne oraz inne komórki. Hamuje syntezę IL-1, IL-6, IL-12 i TNF-α (Glocker et al., 2009; Neurath, 2014). Niedobór IL-10 u myszy skutkował ciężką postacią zapalenia jelit (Glocker et al., 2009).

W jelicie krętym obecna jest ciągła warstwa kępek Peyera, w przeciwieństwie do dwunastnicy i jelita czczego (Obremski et al., 2020), dlatego zdecydowano o zbadaniu stężenia wybranych cytokin właśnie z tego odcinka jelita cienkiego. W kępkach Peyera

znajdują się komórki M, które są zdolne do pobierania antygenu ze światła jelita i prezentowania go komórkom T i B poprzez komórki dendrytyczne. W ten sposób cząsteczki również mikroplastik mogą być prezentowany komórkom układu immunologicznego (Maroilley et al., 2018).

Neurotransmitery i cytokiny uczestniczą w neuroimmunoregulacji poprzez interakcje ze specyficznymi receptorami na komórkach odpornościowych i neuroendokrynnych. Synteza cytokin regulowana jest m. in. dzięki proliferacyjnym lub antyproliferacyjnym właściwościom neuroprzekaźników, które oddziałują na limfocyty błony śluzowej i komórki nabłonka jelitowego (Gonkowski et al., 2015).

5. Hipoteza i cele badawcze

Hipoteza: mikroplastik politereftalanu etylenu może powodowaće zmiany w kodowaniu chemicznym (CART, GAL, nNOS, SP, VAChT i VIP) jelitowego układu nerwowego jelita cienkiego oraz zmiany stężenia cytokin (IL-1β, IL-6, IL-8, IL-10 oraz TNF-α) w jelicie krętym świni domowej (*Sus scrofa domesticus*).

W celu weryfikacji hipotezy przeprowadzono badania oceny wpływu niskiej (0,1 g/dzień) i wysokiej dawki (1 g/dzień) mikroplastiku na kodowanie chemiczne jelitowego układu nerwowego i układu immunologicznego jelita cienkiego świni domowej, jako organizmu modelowego.

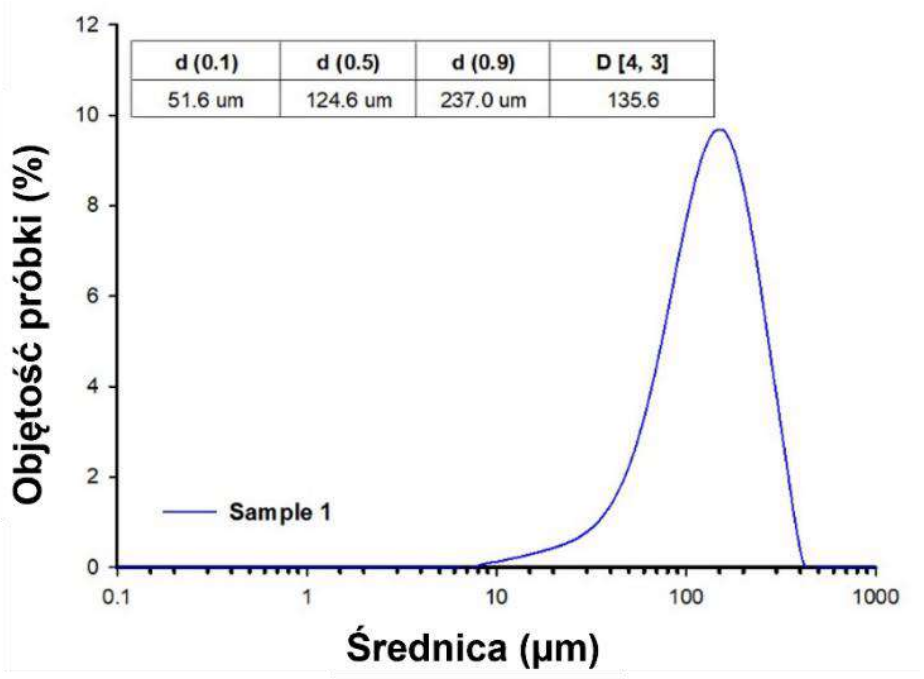
Cel główny został osiągnięty poprzez realizację następujących celów szczegółowych:

1. Określenie odsetka neuronów pozytywnych względem CART, GAL, nNOS, SP, VAChT oraz VIP na terenie dwunastnicy, jelita czczego oraz jelita krętego u świń z grupy kontrolnej.
2. Określenie wpływu MP-PET w dawce 0,1 g/zwierzę/dzień na odsetek neuronów pozytywnych względem CART, GAL, nNOS, SP, VAChT oraz VIP na terenie dwunastnicy, jelita czczego oraz jelita krętego u świni domowej.
3. Określenie wpływu MP-PET w dawce 1 g/zwierzę/dzień na odsetek neuronów pozytywnych względem CART, GAL, nNOS, SP, VAChT oraz VIP na terenie dwunastnicy, jelita czczego oraz jelita krętego u świni domowej.
4. Określenie stężenia IL-1β, IL-6, IL-8, IL-10 oraz TNF-α w ścianie jelita krętego u świń z grupy kontrolnej.
5. Określenie wpływu MP-PET w dawce 0,1 g/zwierzę/dzień na stężenia IL-1β, IL-6, IL8, IL-10 oraz TNF-α w ścianie jelita krętego u świni domowej.
6. Określenie wpływu MP-PET w dawce 1 g/zwierzę/dzień na stężenia IL-1β, IL-6, IL-8, IL-10 oraz TNF-α w ścianie jelita krętego u świni domowej.
7. Przeprowadzenie analizy statystycznej uzyskanych wyników w celu określenia istotności statystycznej zaobserwowanych zmian.

6. Materiały i metody

6.1. Charakterystyka mikroplastiku

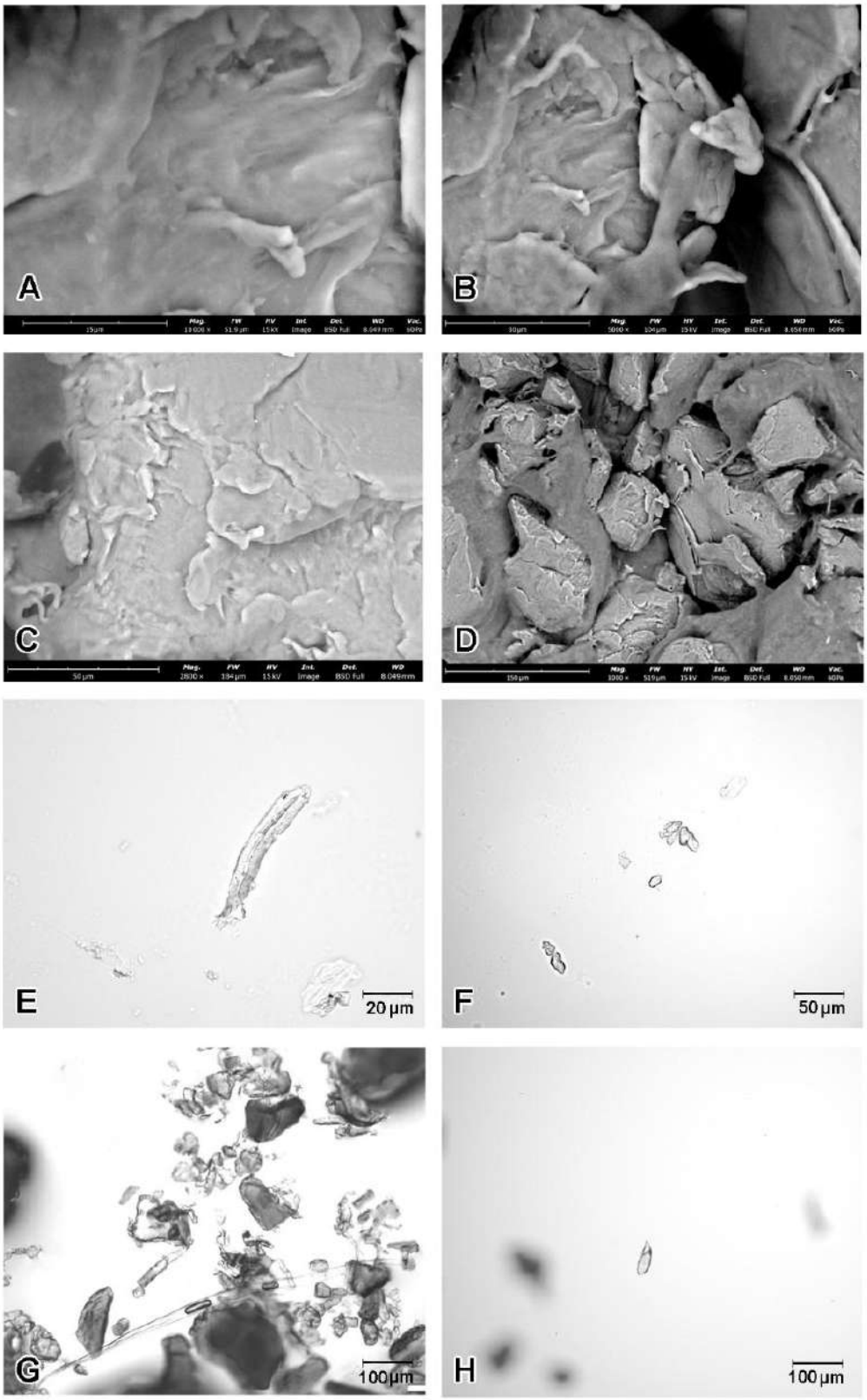
W badaniu wykorzystano półkrystaliczny proszek PET (nr. kat. ES306031/1, Goodfellow Cambridge Ltd., Huntingdon, UK). Według producenta wielkość cząsteczek nie przekraczała 300 µm. Analiza wielkości cząsteczek została wykonana przy użyciu aparatu Mastersizer 2000 (Malvern Instruments Ltd., Malvern, UK) i przystawki Scirocco 2000. Pomiar oparty jest na technice dynamicznego rozpraszania światła. Uzyskane wyniki wskazują, że 10% cząsteczek miało średnicę mniejszą niż 51,6 µm, 50% cząsteczek średnicę mniejszą niż 124,6 µm, a 90% cząsteczek mniejszą niż 237,0 µm. Średnica cząsteczek odnosząca się do całej objętości próbki wynosi średnio 135,6 µm (Rycina 1). Próbka zawierała cząsteczki w przedziale wielkości 7,6 µm – 416,9 µm, z dominującym udziałem cząsteczek o średnicy 158,5 µm.



Rycina 1. Rozkład wielkości cząsteczek. d(0.1) – średnia średnica 10 % cząsteczek, d(0.5) – średnia średnica 50% cząsteczek, d(0.9) – średnia średnica 90% cząsteczek. D [4,3] - średnia średnica cząsteczek całej próbki.

W celu wizualizacji kształtu i powierzchni cząsteczek wykorzystano mikroskop skaningowy Phenom ProX G6 (Thermo Fisher Scientific, Waltham, MA, USA) oraz mikroskop immunofluorescencyjny Zeiss Axio Imager.M2 (Zeiss, Oberkochen, Niemcy).

Wykazano, że cząsteczki stanowiły niejednorodną mieszaninę o różnym kształcie, wielkości, co zostało przedstawione na Rycinie 2.

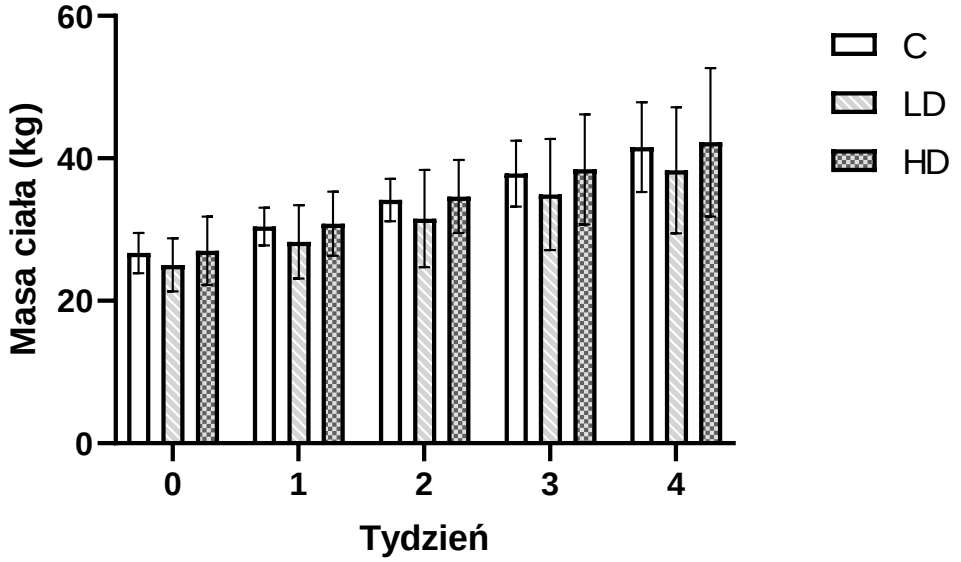


Rycina 1. Mikroplastik. (A-D) obrazy z mikroskopu skaningowego. (E-H) obrazy z mikroskopu immunofluorescencyjnego.

6.2. Zwierzęta

Wykorzystanie zwierząt i zaplanowane procedury doświadczalne zostały zatwierdzone przez Lokalną Komisję Etyczną do Spraw Doświadczeń na Zwierzętach w Olsztynie (Uchwała nr 10/2020 z dnia 26.02.2020).

W doświadczeniu wykorzystano 15 niedojrzałych płciowo (8 tygodniowych) loszek krzyżówki rasy Pietrain x Duroc o początkowej masie ciała około 20 kg (Rycina 3). Zwierzęta już na etapie tygodniowej aklimatyzacji, przy użyciu generatora liczb losowych zostały losowo podzielone na trzy grupy badawcze (n=5): grupa kontrolna (C), grupa otrzymująca niską dawkę MP-PET (LD) oraz grupa otrzymująca wysoką dawkę MP-PET (HD). Loszki utrzymywane były w specjalnie przystosowanych pomieszczeniach Wydziału Medycyny Weterynaryjnej Uniwersytetu Warmińsko-Mazurskiego w Olsztynie w temperaturze 21°C±1°C, wilgotności względnej 57,5%±2,5% oraz 12 godzinnym cyklu dzień-noc. Zwierzęta były karmione dwa razy dziennie, paszą i dawką pokarmową dostosowaną do wieku. Do karmienia i pojenia zostały wykorzystane karmidła wykonane ze stali nierdzewnej. Ważenie loszek odbywało się w tygodniowych interwałach, począwszy od pierwszego dnia aklimatyzacji. Nie wykazano statystycznie istotnych różnic w masie ciała zwierząt pomiędzy grupami (Rycina 3).



Rycina 2. Masa ciała loszek w tygodniowych interwałach.

6.3. Procedura eksperymentalna

Godzinę przed porannym karmieniem zwierzęta z grupy C otrzymywały puste kapsułki żelatynowe. Grupa LD otrzymywała MP-PET w kapsułkach żelatynowych w dawce 0,1 g/zwierzę/dzień, a grupa HD w dawce 1 g/zwierzę/dzień. Zastosowana dawka 0,1, g/dzień/zwierzę została dostosowana do masy loszek na podstawie badania Deng et al. (Deng et al., 2017), podczas gdy dawka 1 g/dzień/zwierzę była dawką 10-krotnie wyższą (opracowana na podstawie logarytmu dziesiętnego (dla log(0,1)= -1; dla log(1)= 0) i miała charakter obciążający). Po 28 dniach zwierzęta zostały poddane eutanazji. Protokół eutanazji oparto na wykorzystaniu atropiny (0,05 mg/kg *i.m.*, Polfa, Warszawa, Polska), a następnie ksylazyny (3 mg/kg *i.m.*, Vet-Agro, Lublin, Polska) i ketaminy (6 mg/kg *i.m.*, Vetoquinol Biowet, Gorzów Wlkp., Polska). Po ok. 20 minutach, gdy loszki były pozbawione świadomości przedawkowano pentobarbital sodowy (0,6 ml/kg, *i. v.*, Biowet, Puławy, Polska). Po potwierdzeniu ustania funkcji życiowych (brak tętna, oddychania, odruchu rogówkowego, brak szmerów sercowych i oddechowych), przystąpiono niezwłocznie do pobrania próbek do dalszych badań. Pobrano fragmenty dwunastnicy (ok. 4 cm, 10 cm od mięśnia zwieracza odźwiernika), jelita czczego (ok. 8 cm, 100 cm od odźwiernika żołądka) oraz jelita krętego (ok. 8 cm, 2 cm przed zastawką krętniczo-kątniczą).

6.4. Barwienia immunofluorescencyjne

W celu utrwalenia próbek pobrane fragmenty jelita cienkiego zostały umieszczone w 4% roztworze paraformaldehydu (pH 7,4) na jedną godzinę, a następnie były płukane przez 3 kolejne dni 0,1 M buforem fosforanowym (pH 7,4) z wymianą buforu co 24 godziny. Po 3 dniach tkanki zostały przeniesione do 18% roztworu sacharozy (pH 7,4) na okres ok. 2 tygodni w temperaturze 4°C. Po utrwaleniu fragmenty jelita cienkiego zatopiono w OCT Tissue-Tek (Sakura Finerek USA, Inc., Torrance, CA, USA) i przy użyciu kriostatu (CM 1860, Leica, Nussloch, Niemcy), pocięto w temperaturze -22°C na skrawki o grubości 14 µm.

Procedura barwienia trwała 2 dni – najpierw w temperaturze pokojowej przez 45 minut suszono szkiełka, następnie przeprowadzono płukanie przy użyciu 0,1 M buforu fosforanowego (PBS, pH 7,4; 3 x 15 minut). Kolejny krok obejmował inkubację z mieszaniną blokującą (10% surowica końska i 0,1% albumina surowicy bydlęcej, 0,1 M PBS, 1% triton X-100, 0,05% thimerosal, oraz 0,01% azydku sodu) przez 1 godzinę w temperaturze pokojowej w wilgotnej komorze. W celu wypłukania mieszaniny blokującej użyto roztwór PBS (3 x 15 minut). Na skrawki jelit nałożono mieszaninę przeciwciał pierwotnych przeciwko markerowi panneuronalnemu – produkt genu proteinowego 9.5 (PGP 9.5) oraz przeciwko jednemu z wybranych antygenów. Kolejnego dnia barwienia wypłukano skrawki roztworem PBS (3 x 15 minut). Na tak przygotowane tkanki na 1 godzinę w wilgotnej komorze nałożono przeciwciała wtórne Alexa Fluor 488 oraz 546. Listę użytych przeciwciał pierwotnych i wtórnych przedstawiono w Tabeli 1. Ostatni etap barwienia polegał na płukaniu roztworem PBS (3 x 15 minut), pokryciu tkanek zbuforowanym glicerolem (pH 8,6) i nałożeniu szkiełka nakrywkowego. Przeprowadzono rutynowe testy kontrolne przeciwciał, aby wykluczyć nieswoiste barwienie. Testy te obejmowały wstępną absorpcję przeciwciał z odpowiednim antygenem, test pominięcia i zastąpienie przeciwciał pierwotnych surowicami nieimmunologicznymi.

Wybarwione fragmenty jelit poddano analizie przy użyciu mikroskopu fluorescencyjnego Zeiss Axio Imager.M2 (Zeiss, Oberkochen, Niemcy) oraz oprogramowania ZEISS ZEN 3.0 (Zeiss, Oberkochen, Niemcy). Od każdego osobnika zliczono 500 neuronów PGP 9.5 pozytywnych z wyraźnie widocznym jądrem w każdym z trzech splotów (MP, OSP i ISP) jako 100% populacji. Następnie poprzez zmianę filtra uwidoczniono i zliczono neurony pozytywne względem badanych substancji i określono jako odsetek neuronów PGP 9.5 dodatnich. Aby uniknąć zliczania tych samych neuronów, odległość pomiędzy fragmentami wynosiła co najmniej 200 µm.

Tabela 1. Lista użytych przeciwciał pierwotnych i wtórnych.

Przeciwciała pierwotne				
Antygen	**Gatunek**	**Stężenie**	**Numer katalogowy**	**Producent**
PGP 9.5	Mysz	1:1000	480012	ThermoFisher Scientific, Waltham, MA, USA
CART	Królik	1:16000	H-003-61	Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA
GAL	Kawia domowa	1:2000	T-5036	Peninsula, San Carlos, CA, USA
nNOS	Królik	1:3000	PA1-033	ThermoFisher Scientific, Waltham, MA, USA
SP	Szczur	1:150	8450-0505	BioRad, Hercules, CA, USA
VAChT	Królik	1:2000	H-V006	Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA
VIP	Królik	1:2000	ab22736	Abcam, Cambridge, Wielka Brytania
Przeciwciała wtórne				
Alexa Fluor 488 anti-mouse IgG (H + L)	Osioł	1:1000	A21202	ThermoFisher Scientific, Waltham, MA, USA
Alexa Fluor 546 anti-rabbit IgG (H + L)	Osioł		A10040	
Alexa Fluor 546 anti-guinea pig IgG (H + L)	Koza		A11074	
Alexa Fluor 546 anti-rat IgG (H + L)	Koza		A11081	

6.5. Badania stężenia cytokin

Fragmenty jelita natychmiast po pobraniu zamrożono w ciekłym azocie i przetrzymywano w -80°C do wykonania analiz. Każda próbka (1 g) została poddana homogenizacji przy użyciu buforu ekstrakcyjnego składającego się z PBS (nr. kat. P4417, Sigma-Aldrich, Saint Louis, MO, USA), inhibitorów proteazy (cOmplete ULTRA Tablets, Mini, EASYpack Protease Inhibitor Cocktail, nr. kat. 05892970001, Sigma-Aldrich, Saint Louis, MO, USA) i homogenizatora (Homogenizator T10 Basic Ultra-Turrax IKA Poland Sp. z o.o., Warszawa, Polska). Następnie odwirowano przy 5000 g przez 60 minut przy użyciu wirówki Eppendorf 5804R (Eppendorf, Hamburg, Niemcy) a uzyskany supernatant stanowił materiał do dalszej analizy przy użyciu komercyjnych zestawów ELISA według instrukcji producenta (Tabela 2).

Wcześniej koncentracja białka została określona za pomocą Qubit Protein Assay (nr. kat. Q33211, ThermoFisher Scientific, Waltham, MA, USA) i Qubit 2.0 Fluorometer (nr. kat. Q32866, ThermoFisher Scientific, Waltham, MA, USA) zgodnie z instrukcją producenta. Pomiary absorbancji wykonano na Multiskan FC Microplate Photometer (ThermoFisher Scientific, Waltham, MA, USA) w dwóch powtórzeniach. Stężenie cytokin w próbach homogenatów porównano do zawartości białka.

Tabela 2. Lista użytych zestawów ELISA.

Antygen	Zestaw ELISA	Numer katalogowy	Producent
IL-1β	Porcine IL-1beta ELISA Kit	orb566534	Biorbyt, Cambridge, Wielka Brytania
IL-6	Porcine IL-6 ELISA Kit	orb566538	
IL-8	Porcine IL-8 ELISA Kit	orb566594	
IL-10	Porcine IL-10 ELISA Kit	orb566526	
TNF-α	Porcine TNF-alpha ELISA Kit	orb566582	

6.6. Analiza statystyczna

Przed przeprowadzeniem właściwej analizy statystycznej sprawdzono założenie liniowości i normalności. Do badania liniowości wygenerowano dwuwymiarowe wykresy rozrzutu analizowanych zmiennych. Założenie normalności zostało potwierdzone za pomocą histogramów i wykresów normalności reszt. Brak wystąpienia homogeniczności wariancji stwierdzano przy wykorzystaniu testu Levene'a. Jednoczynnikowa analiza wariancji (ANOVA) z testem post-hoc Schéffe została wykorzystana w celu wykazania statystycznie istotnych różnic. Różnice pomiędzy grupą C, a LD i HD były rozpatrywane na poziomie $p<0,05$ ($*p<0,05$, $**p<0,01$, $***p<0,001$). Wyniki przedstawiono jako średnia ± standardowy błąd średniej. W opisie wyników analizy statystycznej nie uwzględniono różnic pomiędzy grupą LD a HD. Za zmienną niezależną przyjęto grupę badawczą, a za zmienną zależną odsetek neuronów immunoreaktywnych względem badanych neurotransmiterów i neuropeptydów oraz poziom cytokin. Wyniki poddano analizie statystycznej przy użyciu oprogramowania Statistica 13.3 (TIBCO Software Inc., Palo Alto, USA).

7. Wyniki

7.1. Barwienia immunofluorescencyjne

Immunoreaktywność względem PGP 9.5 oraz badanych neurotransmiterów lub neuropeptydów odnotowano u świń we wszystkich grupach badawczych oraz we wszystkich badanych splotach. W Tabeli 3. przedstawiono w sposób uproszczony zaobserwowane zmiany, które zostały dokładniej są opisane w dalszej części sekcji wyników.

Tabela 3. Odsetek neuronów CART-, GAL-, nNOS-, SP-, VAChT- i VIP-dodatnich w jelicie cienkim. Strzałkami oznaczono zmiany w populacji neuronów pozytywnych względem grupy kontrolnej. - - brak statystycznie istotnych zmian, ↑/↓ - zmiany istotne na poziomie p<0,05, ↑↑/↓↓ - zmiany istotne na poziomie <0,01, ↑↑↑/↓↓↓ - zmiany istotne na poziomie p<0,001.

Grupa	Dwunastnica			Jelito czcze			Jelito kręte		
	MP	OSP	ISP	MP	OSP	ISP	MP	OSP	ISP
					CART				
LD	-	↑	-	↓↓↓	↓↓↓	-	-	↓	↓↓↓
HD	↑↑↑	↑↑↑	↑↑↑	↓↓↓	↓↓↓	↓	-	↓↓↓	↓↓↓
					GAL				
LD	-	-	-	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↓↓	-
HD	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↓↓↓	↓↓↓
					nNOS				
LD	↓↓	-	↓↓	-	-	-	↓↓↓	-	-
HD	↓↓↓	↓↓↓	↓↓↓	↓↓↓	-	↓↓	↓↓↓	↓↓	↓↓↓
					SP				
LD	-	↓↓↓	-	↓↓↓	-	↑↑↑	↑↑↑	↑↑↑	↑↑↑
HD	↓	↓↓↓	↓↓↓	↓↓↓	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑
					VAChT				
LD	-	-	-	-	↓↓	↓	↓↓	↓↓	-
HD	↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓	↓↓↓
					VIP				
LD	-	↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓
HD	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓

7.1.1. Transkrypt regulowany przez kokainę i amfetaminę

Analiza statystyczna wykazała, że w dwunastnicy u grup eksperymentalnych odnotowano zwiększony odsetek neuronów CART-dodatnich w porównaniu do grupy kontrolnej. Niska dawka MP-PET była odpowiedzialna za wywołanie zaobserwowanych zmian jedynie na terenie OSP – 10,25±1,23%; $p<0,05$, a wysoka dawka MP-PET we wszystkich badanych splotach (MP – 21,79±2,11%; OSP – 16,27±1,70%; ISP - 13,06±0,48%; $p<0,001$). W przeciwieństwie do dwunastnicy, w jelicie czczym i krętym odnotowano zmniejszenie populacji neuronów CART-dodatnich. W jelicie czczym zmiany dotyczyły głównie neuronów MP (LD – 4,32±0,37%; HD – 4,95±0,77%; $p<0,001$) oraz ISP (LD – 2,80±0,53%; HD – 2,67±0,26%; $p<0,001$), a w mniejszym stopniu podśluzowego wewnętrznego (HD – 2,19±0,51%; $p<0,05$), gdzie niska dawka MP-PET nie wywołała statystycznie istotnych zmian (2,80±1,34%; $p>0,05$). W jelicie krętym na terenie MP nie odnotowano statystycznie istotnych zmian (LD – 5,30±0,69%; HD - 4,25±0,51%; $p>0,05$). Odsetek neuronów w OSP jelita krętego uległ zmniejszeniu pod wpływem niskiej (3,67±1,23%; $p<0,05$) i wysokiej (2,36±0,83%; $p<0,001$) dawki MP-PET. W obu grupach otrzymujących MP-PET odnotowano znaczne zmniejszenie populacji neuronów CART-dodatnich (LD – 3,81±0,94%; HD – 1,78±0,47%; $p<0,001$). Uzyskane wyniki przedstawiono w Tabeli 4.

*Tabela 4. Odsetek neuronów CART-dodatnich w odcinkach jelita cienkiego świni domowej w grupie kontrolnej (C) oraz grupach otrzymujących niską dawkę (LD) i wysoką dawkę (HD) MP-PET. Wyniki przedstawiono jako średnia ± standardowy błąd średniej. *- p < 0,05, ***- p <0,001 wskazują na statystycznie istotne różnice w odsetku neuronów w grupie LD i HD względem grupy kontrolnej.*

Grupa	Dwunastnica	Jelito czcze	Jelito kręte
		MP	
C	8,71±0,58	9,38±1,64	5,67±0,99
LD	9,90±0,77	4,32±0,37 ***	5,30±0,69
HD	21,79±2,11 ***	4,95±0,77 ***	4,25±0,51
		OSP	
C	7,68±0,86	5,27±1,20	5,23±0,59
LD	10,25±1,23 *	2,80±0,53 ***	3,67±1,23 *
HD	16,27±1,70 ***	2,67±0,26 ***	2,36±0,83 ***
		ISP	
C	4,94±0,59	3,42±1,28	8,62±0,85
LD	6,62±1,21	2,80±1,34	3,81±0,94 ***
HD	13,06±0,48 ***	2,19±0,51 *	1,78±0,47 ***

7.1.2. Galanina

W grupie otrzymującej niską dawkę MP-PET w dwunastnicy nie odnotowano statystycznie istotnych różnic w żadnym z badanych splotów (MP - 9,57±1,18%; OSP - 16,04±1,21%; ISP - 26,66±2,30%; $p>0,05$). W grupie otrzymującej wysoką dawkę zaobserwowano zwiększenie populacji neuronów GAL-dodatnich (MP - 19,79±1,15%; OSP - 29,92±0,95%; ISP - 35,51±1,25%; $p<0,001$). Obie dawki MP-PET miały porównywalny wpływ na odsetek neuronów jelita czczego znajdujących się w MP (LD - 6,63±0,83%; HD - 11,28±1,86%; $p < 0,001$), OSP (LD - 19,24±1,93%; HD - 20,10±2,25%; $p < 0,001$) i ISP (LD - 23,22±3,49%; HD - 21,52±3,25%; $p < 0,001$). Zaobserwowana tendencja w dwunastnicy i jelicie czczym, mianowicie wzrost populacji neuoronów immunoreaktywnych względem galaniny, na terenie jelita krętego była analogiczna jedynie w MP (LD - 8,86±0,92%; HD - 12,13±1,18%; $p < 0,001$). W splotach podśluzowych niska dawka MP-PET zmniejszyła populację tych neuronów tylko w OSP - 12,66±0,37% ($p < 0,01$). Wysoka dawka MP-PET natomiast zmniejszyła odsetek neuronów GAL-dodatnich do 8,68±1,40% ($p < 0,001$) w OSP i 11,19±1,31% ($p < 0,001$) w ISP. Uzyskane wyniki przedstawiono w Tabeli 5.

*Tabela 5. Odsetek neuronów GAL-dodatnich w odcinkach jelita cienkiego świni domowej w grupie kontrolnej (C) oraz grupach otrzymujących niską dawkę (LD) i wysoką dawkę (HD) MP-PET. Wyniki przedstawiono jako średnia ± standardowy błąd średniej. **- p < 0,01, ***- p <0,001 wskazują na statystycznie istotne różnice w odsetku neuronów w grupie LD i HD względem grupy kontrolnej.*

Grupa	Dwunastnica	Jelito czcze	Jelito kręte
		MP	
C	8,87±1,27	3,40±1,08	4,86±0,61
LD	9,57±1,18	6,63±0,83 ***	8,86±0,92 ***
HD	19,79±1,15 ***	11,28±1,86 ***	12,13±1,18 ***
		OSP	
C	16,48±1,45	11,90±1,00	15,84±0,90
LD	16,04±1,21	19,24±1,93 ***	12,66±0,37 **
HD	29,92±0,95 ***	20,10±2,25 ***	8,68±1,40 ***
		ISP	
C	26,37±1,74	12,49±1,30	24,18±1,11
LD	26,66±2,30	23,22±3,49 ***	25,10±1,73
HD	35,51±1,25 ***	21,52±3,25 ***	11,19±1,31 ***

7.1.3. Neuronalna forma syntazy tlenku azotu

Populacja neuronów nitrenergicznych w zależności od badanego odcinka i splotu ulegała redukcji lub nie obserwowano statystycznie istotnych zmian. W MP niska dawka zredukowała odsetek neuronów w dwunastnicy do 39,04±2,27% ($p < 0,01$), a w jelicie krętym do 17,90±1,05% ($p < 0,001$). Natomiast pod wpływem wysokiej dawki we wszystkich odcinkach jelita cienkiego odnotowano zmniejszenie populacji neuronów nNOS-dodatnich w MP (dwunastnica - 26,19±3,50%; jelito czcze - 14,77±3,02%; jelito kręte - 12,78±1,06%; $p < 0,001$).

Liczebność neuronów OSP uległa zmniejszeniu tylko pod wpływem wysokiej dawki MP-PET w dwunastnicy do 19,02±1,51% ($p < 0,001$) oraz jelicie krętym do 8,75±1,00% ($p < 0,01$). Niska dawka MP-PET tylko w dwunastnicy zmniejszyła odsetek neuronów w ISP do 19,79±2,31% ($p < 0,01$). W grupie otrzymującej wysoką dawkę w ISP natomiast odnotowano 16,63±1,65% ($p < 0,001$) neuronów w dwunastnicy, 6,48±1,38% ($p < 0,01$) w jelicie czczym i 6,87±1,52% ($p < 0,001$) w jelicie krętym. Uzyskane wyniki przedstawiono w Tabeli 6.

*Tabela 6. Odsetek neuronów nNOS-dodatnich w odcinkach jelita cienkiego świni domowej w grupie kontrolnej (C) oraz grupach otrzymujących niską dawkę (LD) i wysoką dawkę (HD) MP-PET. Wyniki przedstawiono jako średnia ± standardowy błąd średniej. **-p < 0,01, ***-p < 0,001 wskazują na statystycznie istotne różnice w odsetku neuronów w grupie LD i HD względem grupy kontrolnej.*

Grupa	Dwunastnica	Jelito czcze	Jelito kręte
		MP	
C	43,57±1,44	25,78±2,78	25,74±1,53
LD	39,04±2,27 **	23,47±1,84	17,90±1,05 ***
HD	26,19±3,50 ***	14,77±3,02 ***	12,78±1,06 ***
		OSP	
C	28,91±0,70	12,07±3,16	11,54±1,51
LD	26,68±2,06	12,06±2,33	11,03±0,88
HD	19,02±1,51 ***	11,06±1,82	8,75±1,00 **
		ISP	
C	23,24±0,93	8,76±2,66	13,47±1,79
LD	19,79±2,31 **	7,79±1,87	13,59±1,86
HD	16,63±1,65 ***	6,48±1,38 **	6,87±1,52 ***

7.1.4. Substancja P

Zmniejszenie odsetka neuronów SP-dodatnich zanotowano we wszystkich splotach dwunastnicy oraz MP jelita czczego pod wpływem wysokiej dawki MP-PET. Natomiast zwiększenie wykazano w OSP i ISP jelita czczego i wszystkich trzech splotach jelita krętego. Niska dawka MP-PET wywołała zmiany w MP w jelicie czczym (1,83±0,50%; $p<0,001$) i jelicie krętym (6,99±1,12%; $p<0,001$), w OSP w dwunastnicy (13,94±2,08%; $p<0,001$) i jelicie krętym (24,79±2,24%; $p<0,001$), a w ISP w jelicie czczym (21,96±1,92%; $p<0,001$) i krętym (27,61±2,41%; $p<0,001$). Liczba neuronów SP-dodatnich w grupie otrzymującej wysoką dawkę w dwunastnicy uległa zmniejszeniu do 1,19±0,34% w MP, ($p<0,05$) do 10,67±1,88%; w OSP ($p<0,001$), i do 27,95±2,09% w ISP ($p<0,001$). W jelicie czczym w MP doszło do zmniejszenia populacji do 2,13±0,62% ($p<0,001$), ale w splotach podśluzowych zanotowano już odwrotną tendencję w OSP 17,10±2,52%($p<0,001$) i ISP 30,35±2,15% ($p<0,001$). W jelicie krętym zaobserwowano podobną tendencję, mianowicie w MP doszło do zwiększenia do 8,94±1,62% ($p<0,001$), w OSP do 24,13±1,59% ($p<0,001$) i w ISP do 31,96±1,11% ($p<0,001$). Uzyskane wyniki przedstawiono w Tabeli 7.

*Tabela 7. Odsetek neuronów SP-dodatnich w odcinkach jelita cienkiego świni domowej w grupie kontrolnej (C) oraz grupach otrzymujących niską dawkę (LD) i wysoką dawkę (HD) MP-PET. Wyniki przedstawiono jako średnia ± standardowy błąd średniej. *-p < 0,05, ***-p < 0,001 wskazują na statystycznie istotne różnice w odsetku neuronów w grupie LD i HD względem grupy kontrolnej.*

Grupa	Dwunastnica	Jelito czcze	Jelito kręte
		MP	
C	2,25±0,33	4,97±1,66	2,68±0,39
LD	2,03±0,32	1,83±0,50 ***	6,99±1,12 ***
HD	1,19±0,34 *	2,13±0,62 ***	8,94±1,62 ***
		OSP	
C	19,05±1,86	12,32±1,22	19,07±1,91
LD	13,94±2,08 ***	14,08±1,33	24,79±2,24 ***
HD	10,67±1,88 ***	17,10±2,52 ***	24,13±1,59 ***
		ISP	
C	35,34±1,50	13,30±1,60	21,90±1,68
LD	32,97±3,04	21,96±1,92 ***	27,61±2,41 ***
HD	27,95±2,09 ***	30,35±2,15 ***	31,96±1,11 ***

7.1.5. Pęcherzykowy transporter acetylocholiny

W dwunastnicy odnotowano statystycznie istotne zmiany jedynie pod wpływem wysokiej dawki MP-PET (MP - 12,90±2,95%; $p<0,001$; OSP - 11,76±0,56%; $p<0,001$; ISP - 8,87±1,29%; $p<0,001$). Niska dawka MP-PET zmniejszyła populację neuronów VAChT-dodatnich w jelicie czczym tylko na terenie OSP (29,99±2,69%; $p<0,01$) i ISP (29,76±0,91%; $p<0,05$), a w jelicie krętym w MP (19,67±0,87%; $p<0,01$) i OSP (13,38±0,56%; $p<0,01$). Wysoka dawka natomiast wywoła taki sam efekt we wszystkich badanych splotach jelita czczego (MP - 20,06±1,13%; $p<0,001$; OSP - 19,71±0,60%; $p<0,001$; ISP - 20,30±0,82%; $p<0,001$) i jelita krętego (MP - 9,30±2,04%; $p<0,001$; OSP - 13,62±2,21%; $p<0,01$; ISP - 15,32±1,85%; $p<0,001$). Uzyskane wyniki przedstawiono w Tabeli 8.

*Tabela 8. Odsetek neuronów VAChT-dodatnich w odcinkach jelita cienkiego świni domowej w grupie kontrolnej (C) oraz grupach otrzymujących niską dawkę (LD) i wysoką dawkę (HD) MP-PET. Wyniki przedstawiono jako średnia ± standardowy błąd średniej. *-p < 0,05, **-p < 0,01, ***p < 0,001 wskazują na statystycznie istotne różnice w odsetku neuronów w grupie LD i HD względem grupy kontrolnej.*

Grupa	Dwunastnica	Jelito czcze	Jelito kręte
		MP	
C	16,10±1,84	25,45±1,39	23,09±0,84
LD	14,66±1,14	25,28±0,84	19,67±0,87 **
HD	12,90±2,95 **	20,06±1,13 ***	9,30±2,04 ***
		OSP	
C	16,21±1,33	33,63±1,53	16,77±2,14
LD	15,98±3,14	29,99±2,69 **	13,38±0,56 **
HD	11,76±0,56 ***	19,71±0,60 ***	13,62±2,21 **
		ISP	
C	17,31±2,44	32,91±0,63	20,92±1,68
LD	15,74±3,54	29,76±0,91 *	20,23±1,40
HD	8,87±1,29 ***	20,30±0,82 ***	15,32±1,85 ***

7.1.6. Wazoaktywny peptyd jelitowy

W dwunastnicy niska dawka MP-PET spowodowała zmniejszenie populacji neuronów VIP-dodatnich tylko w splotach podśluzowych (OSP - 14,52±1,18%; $p<0,01$; ISP - 22,65±1,69%; $p<0,001$). Natomiast wysoka dawka MP-PET dodatkowo spowodowała zmniejszenie populacji neuronów VIP-dodatnich na terenie MP (MP - 3,81±0,94%; $p<0,001$; OSP - 8,89±0,54%; $p<0,001$; ISP - 19,47±1,17%; $p<0,001$). Neurony VIP-dodatnie w jelicie czczym uległy redukcji zarówno pod wpływem niskiej (MP - 7,50±0,64%; $p<0,001$; OSP - 13,77±0,78%; $p<0,001$; ISP - 13,41±1,84%; $p<0,001$) i wysokiej dawki MP-PET (MP - 5,23±1,04%; $p<0,001$; OSP - 13,95±1,38%; $p<0,001$; ISP - 9,92±0,60%; $p<0,001$). Analogiczne zmiany zaobserwowano w jelicie krętym pod wpływem niskiej (MP - 7,59±0,71%; $p<0,001$; OSP - 13,86±1,34%; $p<0,001$; ISP - 17,33±1,16%; $p<0,001$) i wysokiej dawki MP-PET (MP - 6,17±0,51%; $p<0,001$; OSP -13,85±1,42%; $p<0,001$; ISP - 18,10±1,54%; $p<0,001$). Uzyskane wyniki przedstawiono w Tabeli 9.

*Tabela 9. Odsetek neuronów VIP-dodatnich w odcinkach jelita cienkiego świni domowej w grupie kontrolnej (C) oraz grupach otrzymujących niską dawkę (LD) i wysoką dawkę (HD) MP-PET. Wyniki przedstawiono jako średnia ± standardowy błąd średniej. **-p < 0,01, ***-p < 0,001 wskazują na statystycznie istotne różnice w odsetku neuronów w grupie LD i HD względem grupy kontrolnej.*

Grupa	Dwunastnica	Jelito czcze	Jelito kręte
		MP	
C	7,42±0,70	15,46±0,98	15,57±0,78
LD	7,98±1,25	7,50±0,64 ***	7,59±0,71 ***
HD	3,81±0,94 ***	5,23±1,04 ***	6,17±0,51 ***
		OSP	
C	17,68±2,35	24,28±1,84	25,21±1,53
LD	14,52±1,18 **	13,77±0,78 ***	13,86±1,34 ***
HD	8,89±0,54 ***	13,95±1,38 ***	13,85±1,42 ***
		ISP	
C	29,93±2,47	26,37±2,14	27,38±1,47
LD	22,65±1,69 ***	13,41±1,84 ***	17,33±1,16 ***
HD	19,47±1,17 ***	9,92±0,60 ***	18,10±1,54 ***

7.2. Badanie stężenia cytokin

Analiza stężenia IL-1β, IL-6, IL-8, IL-10 oraz TNF-α nie wykazała statystycznie istotnych różnic pomiędzy grupami otrzymującymi MP-PET a grupą kontrolną (p>0,05). Interleukina 1β oraz interleukina 8 charakteryzowały się największym stężeniem we wszystkich grupach badawczych. Z przebadanych cytokin, najniższe stężenie odnotowano przy badaniu TNF- α. Uzyskane wyniki przedstawiono w Tabeli 10.

Tabela 10. Wyniki stężenia (pg/mg białka) IL-1β, IL-6, IL-8, IL-10 oraz TNF-α w jelicie krętym. Wyniki przedstawiono jako średnia ± standardowy błąd średniej.

Cytokiny	**Grupa**		
	C	**LD**	**HD**
IL-1β	487,95±56,51	602,83±51,12	511,02±92,93
IL-6	186,59±21,33	141,39±11,90	273,93±55,50
IL-8	438,18±31,44	460,32±41,10	615,34±143,42
IL-10	193,75±19,88	250,17±23,73	253,57±40,49
TNF-α	143,77±17,59	137,31±9,12	249,99±52,46

8. Dyskusja

Przedstawione wyniki potwierdzają adaptację jelitowego układu nerwowego na doustną ekspozycję dwóch dawek MP-PET, wyrażoną poprzez zmiany ilościowe w populacjach neuronów CART-, GAL-, nNOS-, SP-, VAChT- oraz VIP-dodatnich. Stopień nasilenia zmian różni się w zależności od badanego splotu oraz odcinka jelita cienkiego. Należy zaznaczyć, że przedstawione wyniki ukazują jedynie efekt ekspozycji na MP-PET, nie wyjaśniając mechanizmu stojącego za wywołanymi zmianami. Są to pierwsze badania ukazujące wpływ mikroplastiku PET na neurony jelitowego układu nerwowego

Analizując wpływ dawki MP-PET na neurony ENS, można zaobserwować, że dawka 0,1 g/dzień nie wywołuje zmian w populacji neuronów immunoreaktywnych względem wszystkich badanych neurotransmiterów, w przeciwieństwie do dawki 1 g/dzień (jedyny wyjątek - neurony nNOS-dodatnie jelita czczego, OSP).

Doustna ekspozycja na MP-PET przyczyniła się do redukcji neuronów immunoreaktywnych względem nNOS, VAChT oraz VIP, zaobserwowanej we wszystkich splotach ENS badanych odcinków jelita cienkiego. Neurony CART-, GAL- oraz SP-pozytywne natomiast nie wykazały analogicznej tendencji. Zanotowano różnice pomiędzy badanymi odcinkami jelita cienkiego (CART), ale również pomiędzy splotami w tym samym odcinku jelita cienkiego (GAL oraz SP).

Badania m. in. nad bisfenolem A potwierdzają, że wyniki uzyskane dla tego samego neurotransmitera mogą być różne w zależności od badanego splotu oraz odcinka przewodu pokarmowego (Szymańska et al., 2018; Szymańska & Gonkowski, 2018). Różnice dotyczące splotów mogą być związane z faktem mechanicznego oddziaływania mikroplastiku, mianowicie, bardziej wyrażone uszkodzenia splotu nerwowego mogą być obecne bliżej światła jelita (Balemba et al., 2001). Innym wytłumaczeniem jest selektywność względem badanego splotu i funkcji, jakie pełni - w uproszczeniu splot mięśniowy odpowiedzialny jest głównie za funkcje motoryczne, a splot podśluzowy za funkcje sekrecyjne (Bulc et al., 2018). Nieprawidłowości w funkcjonowaniu splotów mogą determinować charakter objawów klinicznych takich jak zaburzenia trawienia i wchłaniania składników pokarmowych lub wystąpienie biegunek.

Bazując na obecnej literaturze, nie można potwierdzić, że każdy mikroplastik wywołuje takie same zmiany (Renzi et al., 2019). Renzi et al. stwierdzili, że efekty wywierane na organizm mogą być podobne lub rozbieżne między różnymi plastikami (Renzi et al., 2019). Badania porównujące PET i polietylen wykazały, że PET może wykazywać potencjalnie

większe właściwości toksyczne, np. ze względu na swoje silniejsze zdolności utleniające i zdolność do dysocjacji par nukleozydów guanina–cytozyna, co z kolei może wpływać na replikację DNA (Martínez & Barbosa, 2024).

Jedyne dane dotyczące wpływu mikroplastiku na badane neurotransmitery i neuropeptydy dotyczą neurotransmisji cholinergicznej (Sofield et al., 2024). Neurotoksyczność mikroplastiku powiązana jest z obniżeniem aktywności acetylocholinoesterazy – enzymu rozkładającego ACh na cholinę i resztę kwasu octowego (Li et al., 2023; Prüst et al., 2020). Istnieją przesłanki, że VAChT może stanowić marker procesów regeneracyjnych zakończeń neuronów ruchowych (Maeda et al., 2004). Biorąc pod uwagę, że mikroplastik może doprowadzić do degeneracji komórek nerwowych (Aliakbarzadeh et al., 2023), obniżenie populacji neuronów VAChT-dodatnich, może być następnym dowodem na uszkodzenie neuronów i stanowić kolejny mechanizm neurotoksyczności mikroplastiku. Zaobserwowano również, że podczas uszkodzenia neuronów może dochodzić do zwiększenia wydzielania galaniny (Nezami & Srinivasan, 2010), co jest zauważalne w przedstawionych badaniach na terenie dwunastnicy oraz jelita czczego. W celu potwierdzenia tej tezy należałoby jednak oznaczyć markery uszkodzenia komórek (Koníčková et al., 2022). Poziom neurotransmiterów może być również skorelowany z intensywnością stanu zapalnego – im bardziej zaawansowany stan, tym ich poziom może być niższy (Balemba et al., 2001). Acetylocholina jak już wcześniej wspomniano jest jednym z głównych czynników regulujących motorykę przewodu pokarmowego, przepływ krwi i funkcje wydzielnicze (Harrington et al., 2010). Wykazuje również działanie przeciwzapalne poprzez obniżanie poziomu cytokin prozapalnych (Van Der Zanden et al., 2009). Redukcja neuronów VAChT-dodatnich obserwowana była u świń eksponowanych na działanie bisfenolu A (Szymańska, Makowska, et al., 2018) lub glifosatu (Bulc et al., 2024).

Funkcja CART na terenie ENS nie została jeszcze dobrze zbadana. U świń potwierdzono, że CART na terenie ENS występuje głównie w żołądku, dwunastnicy i jelicie czczym (Ekblad, 2006). Aktualne dane wskazują, że pełni on funkcje neuroprotekcyjne, ale również działa inhibitująco na opróżnianie żołądka i sekrecję kwasu żołądkowego (Okumura et al., 2000). Neuroprotekcja może być powiązana z działaniem antyoksydacyjnym (Mao et al., 2013), a jak już wcześniej wspomniano wywołanie stresu oksydacyjnego jest jednym z głównych mechanizmów działania mikroplastiku (Płuciennik et al., 2024). Liczebność neuronów CART-dodatnich jest stosunkowo niska, co może świadczyć o fakcie, że CART nie odgrywa znaczącej roli w odpowiedzi ENS na mikroplastik w jelicie czczym i krętym, w przeciwieństwie do dwunastnicy. Pod wpływem bisfenolu A (Szymańska

& Gonkowski, 2018) lub w przebiegu choroby Huntingtona (van der Burg et al., 2011) zaobserwowano zmniejszenie populacji neuronów CART-pozytywnych w ENS, w przeciwieństwie do indometacyny (Czajkowska & Całka, 2020), akrylamidu (Palus et al., 2018) lub glifosatu (Palus et al., 2023).

Odsetek neuronów VIP-dodatnich często wzrasta pod wpływem czynników zapalnych (Ekblad & Bauer, 2004) i spada np. w chorobie Parkinsona (Giancola et al., 2017). Brak wzrostu odsetka neuronów nNOS- i VIP-pozytywnych może sugerować fakt, że żaden z tych neuroprzekaźników nie bierze udziału w neuroprotekcji neuronów w ENS pod wpływem MP-PET. W hodowlach komórkowych wykazano, że zahamowanie aktywności VIP i NO zwiększa śmiertelność neuronów (Ekblad & Bauer, 2004). Zarówno VIP i NO rozluźniają mięśnie gładkie jelit oraz hamują wydzielanie na terenie przewodu pokarmowego (Makowska & Gonkowski, 2024). Zmniejszenie populacji neuronów VIP-dodatnich obserwowano u świń po narażeniu na bisfenol A (Szymańska & Gonkowski, 2018) i lipopolisacharyd (Rytel et al., 2021). Jednak większość stanów zapalnych skutkuje wzrostem aktywności VIP ze względu na jego właściwości immunomodulacyjne i neuroprotekcyjne (Morell et al., 2012). Co ciekawe, w przebiegu choroby zapalnej jelit obserwowano wzrost lub spadek VIP, w zależności od badania (Vasina et al., 2006), co potwierdza złożoność układu jakim jest ENS.

Zmienność liczby neuronów nNOS-dodatnich jest zjawiskiem często obserwowanym w zaburzeniach żołądkowo-jelitowych. Tlenek azotu jest uważany za jeden z głównych czynników hamujących, powodujących rozluźnienie mięśni jelit, hamowanie wydzielania hormonów jelitowych i regulację transportu wody i elektrolitów (Szymańska, Calka, et al., 2018; Takahashi, 2003). Gautam et al. przeprowadzili badanie wykorzystujące mikroplastik polietylenowy i linie komórek ludzkich, w tym Caco-2 (komórki nabłonka jelitowego) (Gautam et al., 2022). Uzyskane wyniki wskazują, że mikroplastik spowodował zwiększenie stężenia NO w liniach komórkowych Caco-2, co nie znajduje potwierdzenia w niniejszych badaniach. Może to stanowić kolejny dowód, że rodzaj polimeru determinuje charakter obserwowanych efektów.

Badania innych autorów sugerują, że mikroplastik wpływa stymulująco głównie na wzrost ekspresji SP oraz GAL, co może świadczyć o ich udziale w nocycepcji oraz mechanizmach przeciwbólowych (Wiesenfeld-Hallin & Xu, 2001). Galanina może wpływać na motorykę przewodu pokarmowego poprzez hamowanie uwalniania ACh i SP z pobudzających neuronów ruchowych (Nezami & Srinivasan, 2010). Regulacja motoryki jest o tyle istotna, że zahamowanie aktywności skurczowej przewodu pokarmowego może być zjawiskiem potencjalnie niepożądanym, który może sprzyjać

przenikaniu nanocząsteczek do krwioobiegu. W trakcie trwania doświadczenia nie odnotowano pojawienia się biegunki, zaparć, zmniejszonego apetytu u zwierząt czy zmniejszenia przyrostów masy ciała.

Galanina może wpływać na motorykę przewodu pokarmowego poprzez hamowanie wydzielania ACh i SP za pośrednictwem pobudzających receptorów GAL-R1 (Nezami & Srinivasan, 2010). Spadek aktywności SP odnotowano po długotrwałym narażeniu świń na naproksen (Czajkowska et al., 2020) oraz u szczurów po podaniu środków powodujących owrzodzenia (Renzi et al., 1991), z zespołem dysfunkcji wielu narządów (Xie et al., 2015) i cukrzycą (Ballmann & Conlon, 1985). Wiązano to z rozkurczem mięśni jelit prowadzącym do zaburzeń motoryki lub z powstawaniem owrzodzeń dwunastnicy. U osób z zaparciami (Tzavella et al., 1996) lub cukrzycą zaobserwowano spadek poziomu SP w błonie śluzowej odbytu (Lysy et al., 1997) i żołądka (Iwasaki et al., 2006). Wzrost aktywności SP można zaobserwować znacznie częściej pod wpływem różnych czynników wywołujących stan zapalny w jelitach (Czajkowska & Całka, 2020; Szymańska & Gonkowski, 2018).

Wyniki badań pokazują, że MP-PET wpływa na geny związane z chorobami cywilizacyjnymi (insulinooporność, otyłość, choroby układu krążenia i nowotwory) poprzez miRNA (Mierzejewski et al., 2023). Ponadto wykazano zależność pomiędzy chorobami neurodegeneracyjnymi, jak choroba Alzheimera lub Parkinsona, a mikroplastikiem (Jeong et al., 2024; Wang et al., 2024). Mechanizm działania związany jest z uszkodzeniem lub redukcją neuronów, indukowaniem śmierci komórek lub agregacją α-synukleiny (Sofield et al., 2024). Powinno to budzić niepokój, ponieważ mikroplastik jest ksenobiotykiem, na który ludzie są powszechnie narażeni, a jego negatywne skutki nie zostały jeszcze w pełni poznane.

Zaistniałe różnice pomiędzy różnymi odcinkami jelita cienkiego mogą wynikać z odmiennych właściwości fizykochemicznych MP-PET i/lub badanych neuroprzekaźników w zależności od odcinka przewodu pokarmowego. Badania potwierdzają, że proces trawienia może powodować zmiany właściwości fizykochemicznych, w tym uwalnianie nanocząsteczek lub substancji chemicznych (Krasucka et al., 2022; Peters et al., 2022). Cząsteczka, która jest pasażowana przez przewód pokarmowy może wykazywać inne cechy morfologiczne niż przed pasażem (zmiana rozmiaru, kształtu, właściwości chemicznych). Analiza histologiczna wykazała, że zmiany takie jak przekrwienie i skrócenie kosmków jelitowych były bardziej wyrażone w dwunastnicy, a najmniej w jelicie krętym, potwierdzając zależność destrukcyjnego działania mikroplastiku w zależności od odcinka przewodu pokarmowego.

Można zatem założyć, że w dwunastnicy ważniejszy może być aspekt fizyczny oddziaływania MP-PET (wielkość cząsteczek), a w jelicie czczym i krętym aspekt chemiczny i postępujące dalsze jego rozdrabnianie.

Należy zaznaczyć, że ENS jest układem, który dąży do utrzymania homeostazy w jelitach. Nie można wykluczyć, że zaobserwowane w przedstawionych badaniach zmiany wynikają z ostrej toksyczności i dopiero po dłuższym czasie ENS wraca do stanu wyjściowego. Potwierdzeniem tej tezy może być badanie nad doradą (*Sparus aurata*), które wykazało, że powrót do homeostazy po ekspozycji na mikroplastik trwał 30 dni od zaprzestania jego podawania (Hoyo-Alvarez et al., 2022). Dlatego przyszłe badania należałoby rozszerzyć o dłuższą oraz przerywaną ekspozycję na mikroplastik. Biorąc pod uwagę, że prawdopodobne narażenie na nano- i mikroplastik w życiu codziennym nie jest takie samo każdego dnia, daje to możliwość płynnego dostosowania się ENS do tego ksenobiotyku.

Ze względu na prozapalne właściwości mikroplastiku (Pulvirenti et al., 2022), można było przypuszczać, że dojdzie do wzrostu stężenia badanych cytokin prozapalnych. Uzyskane wyniki wskazują na pewne tendencje wzrostowe, mianowicie można zaobserwować, że wraz ze wzrostem dawki MP-PET wzrasta stężenie TNF-α, IL-6 i IL-8, przy czym wartość p pozostaje na poziomie statystycznie nieistotnym ($p>0,05$). Może to wskazywać na fakt, że przyjęte dawki są zbyt niskie, aby wywołać statystycznie istotny efekt lub liczebność grupy jest czynnikiem limitującym, za czym przemawiają dość wysokie wartości standardowego błędu średniej. W swojej pracy Pulvirenti et al. podsumowali dane z różnych badań na temat wpływu mikroplastiku na indukcję stanu zapalnego (Pulvirenti et al., 2022). Stwierdzono, że w przypadku badanych cytokin dochodziło do wzrostu lub braku wystąpienie różnic.

Uzyskane wyniki własne jak i dane literaturowe trudno przenieść na warunki rzeczywiste z kilku względów. Główny problem związany z badaniem wpływu mikroplastiku na różnych modelach badawczych dotyczy dawki - zastosowane dawki doświadczalne często są dawkami przeszacowanymi. Sprecyzowanie ilości spożywanych cząsteczek jest trudne i stanowi wyzwanie dla badaczy. Faktyczne oszacowanie ekspozycji na mikroplastik jest uzależnione od kilku czynników takich jak preferencje żywieniowe, wiek, płeć czy miejsce zamieszkania (Cox et al., 2019; Senathirajah et al., 2021). Według Senathirajah et al. ludzie średnio spożywają 0,1-5 g mikroplastiku/tydzień (Senathirajah et al., 2021). Cox et al. natomiast wskazują, że jest to od 39000 do 52000 cząsteczek/rok w zależności od wieku i płci (Cox et al., 2019). Porównując jednak różne dawki zaprezentowane w literaturze (np. 150 mg/kg (Zou et al., 2024)), zastosowanie 0,1 g/dzień wydaje się być bardziej

realistyczne, choć i tak jest dość duże. Dawka 1 g/dzień stanowi 10-krotność niskiej dawki i można ją określić jako dawkę uderzeniową lub toksyczną, przy której spodziewano się zaobserwowania negatywnego efektu ekspozycji na MP-PET.

Dodatkowo zarówno ludzie i zwierzęta są narażeni na mieszaninę różnych cząsteczek, a nie tylko jeden rodzaj pierwotnego mikroplastiku. Mikroplastik środowiskowy może być wektorem zanieczyszczeń takich jak metale ciężkie czy chorobotwórcze drobnoustroje (Naik et al., 2019). Potencjalnie to właśnie one mogą stanowić większe zagrożenie niż sam mikroplastik. Badania potwierdzają również, że mniejsze cząsteczki stanowią poważniejsze zagrożenie niż większe cząsteczki (Prata et al., 2022; Pulvirenti et al., 2022), jednak nie określają one jasno wartości granicznej.

Na podstawie dostępnej literatury można wysnuć wniosek, że stosunek powierzchni ciała do ilości przyjętych cząsteczek determinuje potencjalne negatywne skutki, które częściej są obserwowane u mniejszych organizmów jak np. skorupiaki i ryby. Również badania na myszach i szczurach donoszą o szkodliwych skutkach. Z drugiej strony w literaturze brakuje badań przeprowadzonych na innych modelach badawczych, zwłaszcza ssakach innych niż gryzonie (Banerjee & Shelver, 2021).

Przedstawione badania posiadają pewne ograniczenia. Badania tkanek od zwierząt towarzyszących potwierdziły obecność mikroplastiku, ale na niskim poziomie, w tym właśnie obecność PET (Prata et al., 2022). Uwzględniając wszechobecność mikroplastiku, nie jest możliwe zastosowanie modeli badawczych, które stanowiłyby rzeczywistą kontrolę negatywną. Pasza używana do karmienia zwierząt nie była badana pod kątem identyfikacji rodzaju i ilości mikrocząsteczek plastiku, jednak wszystkie zwierzęta były karmione tą samą paszą, w celu zapewnienia jednakowych warunków. Aparatura badawcza oraz materiały używane podczas badań wykonane są z tworzyw sztucznych, które mogą być źródłem kontaminacji, jednak ten aspekt jest ważniejszy w badaniach identyfikujących i określających ilość cząsteczek w badanej próbce, niż efekty wywołane przez mikroplastik.

Wielkość próby badawczej (n=5) stanowi kolejne ograniczenie przeprowadzonego badania. W związku z brakiem możliwości użycia większej liczby zwierząt, w analizie statystycznej skupiono się na wartościach *p,* jako wyznaczniku istotności statystycznej, pomijając moc testu. Wykorzystanie większej grupy badawczej pozwoliłoby w przyszłości zminimalizować indywidualne cechy osobników w analizie statystycznej.

W przedstawionym modelu badawczym skupiono się na stężeniu wybranych cytokin wyłącznie w jelicie krętym. Przyszłe badania powinny być rozszerzone o wykorzystanie

pozostałych fragmentów jelita cienkiego w celu uzyskania holistycznego wglądu na wpływ mikroplastiku na przewód pokarmowy.

Kolejny aspekt interpretacji i porównania uzyskanych wyników z innymi danymi dotyczącymi zarówno cytokin jak i ENS, związany jest z metodologiczną stroną prowadzenia badań. Stosowane są różne modele badawcze (warunki *in vitro*/*in vivo*, gatunek modelu zwierzęcego, wielkość oraz rodzaj cząsteczek, dawka i czas narażenia oraz metody badawcze), które utrudniają rzetelne analityczne porównania. Należy zauważyć, że ENS jest złożonym układem biologicznym, a przedstawione wyniki stanowią jedynie fragment wiedzy na temat wpływu MP-PET na świnię domową i nie przedstawiają innych potencjalnych mechanizmów, które mogą odpowiadać za utrzymanie homeostazy całego organizmu (wykraczających również poza ENS).

Podsumowując uzyskane wyniki oraz dane literaturowe można stwierdzić, że mikroplastik stanowi zagrożenie dla środowiska, zwierząt i ludzi. Efekt oddziaływania zależny jest od kilku czynników, ale jednym z najważniejszych jest dawka. Niewątpliwie jest to obszar, który wymaga prowadzenia dalszych badań, zwłaszcza w zakresie długofalowego oddziaływania mikroplastiku na organizmy zwierząt i ludzi.

9. Aspekt praktyczny

Wyniki badań nad wpływem MP-PET na układ nerwowy jelita cienkiego świni domowej, wskazują na możliwości ich praktycznego zastosowania, zarówno w weterynarii, jak i medycynie oraz toksykologii środowiskowej.

Wymaga to jednak powtórzenia badań z zastosowaniem innych tworzyw sztucznych oraz dawek, w celu określenia podobieństw oraz różnic w uzyskanych wynikach. W celu maksymalizacji wykorzystania tkanek (zgodnie z założeniami zasady 3R dotyczącej wykorzystania zwierząt w badaniach naukowych), podczas pobierania materiału biologicznego zabezpieczono różne próbki (np. przysadka, trzustka, nerki, wątroba, jajniki, krew, kał), które będą przedmiotem dalszych badań. Takie działanie pozwoli na kompleksowe zbadanie oddziaływania mikroplastiku PET na organizm świni domowej, a w konsekwencji potencjalnego wpływu na ludzi.

Uzyskane wyniki mogą posłużyć w opracowaniu wzorca zmian w zależności od dawki oraz rodzaju mikroplastiku, co może stanowić klucz do identyfikacji zanieczyszczenia paszy/wody/środowiska mikroplastikiem. Pozwoli to również określić narządy najbardziej narażone na mikroplastik, które później mogą być pobierane podczas próbkobrania, w tzw. *screeningu*.

Określenie narządów najbardziej podatnych na doustną ekspozycję mikroplastiku może przyczynić się do ustalenia schematów diagnostycznych, w celu określenia poziomu zanieczyszczenia mikroplastikiem na badanym obszarze/fermie.

W medycynie określenie zmian w populacjach neuronów w jelitowym układzie nerwowym lub zmian w stężeniach cytokin może stanowić podstawę do wytypowania markerów określających wzmożoną ekspozycję na mikroplastik u ludzi lub być elementem terapii celowanej np. poprzez modulację aktywności wybranych cytokin lub neurotransmiterów.

Wykazanie negatywnego wpływu mikroplastiku na środowisko, ludzi i zwierzęta w myśl koncepcji „One Health” może również zmobilizować kraje UE i inne państwa do zrewidowania regulacji prawnych w celu ulepszenia gospodarki odpadami oraz wykorzystania tworzyw sztucznych w celu zminimalizowania zanieczyszczenia środowiska mikroplastikiem. Istotnym aspektem powinno być także określenie najwyższego dopuszczalnego poziomu pozostałości cząsteczek mikroplastiku w paszach i w produktach pochodzenia zwierzęcego, w celu maksymalizacji bezpieczeństwa łańcucha żywnościowego.

10. Wnioski

1. Efekt działania mikroplastiku zależny jest od jego dawki.
2. Wpływ doustnej 28 dniowej ekspozycji mikroplastiku politereftalanu etylenu w dawce 0,1g/dzień na neurony jelitowego układu nerwowego różni się w zależności od badanego neurotransmitera, splotu nerwowego, odcinka jelita cienkiego świni domowej.
3. Doustna 28 dniowa ekspozycja na mikroplastik politereftalanu etylenu w dawce 1g/dzień przyczynia się do redukcji neuronów immunoreaktywnych względem VIP, VAChT i nNOS w jelitowym układzie nerwowym jelita cienkiego świni domowej.
4. Wpływ doustnej 28 dniowej ekspozycji na mikroplastik politereftalanu etylenu w dawce 1g/dzień na neurony immunoreaktywne względem CART, GAL i SP w jelitowym układzie nerwowym jelita cienkiego świni domowej jest odmienny w zależności od badanego splotu nerwowego oraz odcinka jelita cienkiego.
5. Doustna 28 dniowa ekspozycja na mikroplastik politereftalanu etylenu w dawce 0,1g/dzień i 1 g/dzień nie wpłynęła na stężenie IL-1β, IL-6, IL-8, IL-10 oraz TNF-α w jelicie krętym świni domowej.
6. Uzyskane wyniki wskazują na potrzebę prowadzenia dalszych badań wpływu mikroplastiku na organizm świni domowej.

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11. Oświadczenia współautorów wraz z publikacjami wchodzącymi w skład rozprawy doktorskiej

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w sprawie nadania stopnia doktora

Olsztyn 15.01.2025
(miejscowość, data)

Ismena Gałęcka
(imię i nazwisko kandydata)
Weterynaria
(dyscyplina naukowa)

Przewodniczący Rady Naukowej Dyscypliny Weterynaria
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prof. dr hab. Tomasz Maślanka

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Oświadczam, że w pracach pod tytułem

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Gałęcka, I., & Całka, J. (2024). Oral Exposure to Microplastics Affects the Neurochemical Plasticity of Reactive Neurons in the Porcine Jejunum. Nutrients, 16(14), 2268.

Gałęcka, I., & Całka, J. (2024). Microplastic and the Enteric Nervous System: Effect of PET Microparticles on Selected Neurotransmitters and Cytokines in the Porcine Ileum. Int. J. Mol. Sci., 25(21), 11645

mój wkład merytoryczny w ich przygotowanie polegał na: opracowaniu koncepcji, wykonaniu części eksperymentalnej, opracowaniu i interpretacji wyników tych prac, pozyskaniu finansowania, przygotowaniu manuskryptów i odpowiedzi na recenzje.

Ismena Gałęcka
(podpis kandydata)

Załącznik nr 5 do Załącznika nr 1
do Uchwały Nr 362
Senatu Uniwersytetu Warmińsko-Mazurskiego w Olsztynie
z dnia 27 czerwca 2023 roku
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mój wkład merytoryczny w jej przygotowanie polegał na: wykonaniu części eksperymentalnej, wykonaniu wizualizacji wyników oraz przygotowaniu części manuskryptu poświęconej badaniom histologicznym.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. wet. Ismenę Gałęcką, jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów naukowych opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład kandydata w 80% polegający na zaprojektowaniu doświadczenia i przeprowadzeniu go, wykonaniu pracy laboratoryjnej, opracowaniu i interpretacji wyników, pozyskaniu finansowania, przygotowaniu manuskryptu i odpowiedzi na recenzję.

Natalia Szyryńska
podpis

Załącznik nr 5 do Załącznika nr 1
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OŚWIADCZENIE
współautora

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mój wkład merytoryczny w jej przygotowanie polegał na: opracowaniu metodologii i koncepcji badań oraz korekcie manuskryptów.

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Jarosław Całka
podpis

Particle and Fibre Toxicology

RESEARCH

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Influence of polyethylene terephthalate (PET) microplastic on selected active substances in the intramural neurons of the porcine duodenum

Ismena Gałęcka[1,3*], Natalia Szyryńska[2] and Jarosław Całka[3]

Abstract

Background Currently, society and industry generate huge amounts of plastics worldwide. The ubiquity of microplastics is obvious, but its impact on the animal and human organism remains not fully understood. The digestive tract is one of the first barriers between pathogens and xenobiotics and a living organism. Its proper functioning is extremely important in order to maintain homeostasis. The aim of this study was to determine the effect of microplastic on enteric nervous system and histological structure of swine duodenum. The experiment was carried out on 15 sexually immature gilts, approximately 8 weeks old. The animals were randomly divided into 3 study groups (n = 5/group). The control group received empty gelatin capsules once a day for 28 days, the first research group received daily gelatin capsules with polyethylene terephthalate (PET) particles as a mixture of particles of various sizes (maximum particle size 300 µm) at a dose of 0.1 g/animal/day. The second study group received a dose ten times higher—1 g/animal/day.

Results A dose of 1 g/day/animal causes more changes in the enteric nervous system and in the histological structure of duodenum. Statistically significant differences in the expression of cocaine and amphetamine regulated transcript, galanin, neuronal nitric oxide synthase, substance P, vesicular acetylcholine transporter and vasoactive intestinal peptide between control and high dose group was noted. The histopathological changes were more frequently observed in the pigs receiving higher dose of PET.

Conclusion Based on this study it may be assumed, that oral intake of microplastic might have potential negative influence on digestive tract, but it is dose-dependent.

Keywords Enteric nervous system (ENS), Neurotransmitter, Gastrointestinal tract, Swine, Plastic, Cocaine and amphetamine regulated transcript, Galanin, Neuronal nitric oxide synthase, Substance P, Vesicular acetylcholine transporter, Vasoactive intestinal peptide

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Background

Microplastic

Currently, society and industry generate huge amounts of plastics worldwide, including polyethylene, polypropylene, polyurethane, polyvinyl chloride, polyethylene terephthalate, polystyrene and polyamide. Each year, humanity increases the production of plastics, as exemplified by the value of the plastics market, whose value for 2022 was USD 609.01 billion, and the predicted value for 2030 will be approximately USD 811.57 billion [1]. Plastics are used massively in every aspect of life, from food storage and clothing production to children's toys. Plastics are closely related to the continuous generation of waste by-products and production residues. The above characteristics and applications are closely related to the presence of microplastics. Microplastic present in the environment can be divided, depending on its origin, into primary (produced in such a form) and secondary (formed as a result of an impact on plastic). Hartmann et al. proposed a plastic debris classification system based on (I) chemical composition, (II) physical state, (III) solubility, (IV) size, (V) shape and structure, (VI) color and (VII) origin [2]. According to the aforementioned classification, plastic particles from 1 to 1000 µm should be considered microplastics [2]. The European Food Safety Authority (EFSA) report defines particle sizes between 0.1 and 5000 µm [3] and Frias et al. between 1 and 5000 µm [4]. The information provided indicates the need to standardize the terminology, to avoid misunderstandings or misleading public opinion and the consumers. The massive use of plastics in practically every sphere of human life contributes to the release of microplastics into water [5–7], soil [8] and air [5, 9]. The COVID-19 pandemic and the large-scale use of personal protective equipment (such as masks and gloves) resulted in an increase in the number of microparticles in the environment [10, 11]. The plastic for food and beverages packaging promotes their entry into the digestive tract [12–14]. Plastic waste, regardless of its characteristics, has become a problem that civilization has to face. For example, the European Union, as part of the European Green Deal, plans to reduce the landfilling of plastic waste to 55% by 2030. Despite the undertaken initiatives, the ubiquity of microplastics in the environment is obvious, but their influence on animals and humans is still not fully understood [15–17]. The scientific problem mentioned earlier depends on several factors, such as the type (polystyrene, polyethylene terephthalate, polyethylene, polyvinyl chloride, etc.), particle size (macroplastic, microplastic or nanoplastic), route of entry into the body (aerogenic, transdermal, oral), exposure time, research model or dose [17–19].

The enteric nervous system

The digestive tract is one of the first barriers between pathogens, xenobiotics and living organisms. Its proper functioning is extremely important in order to maintain homeostasis, but to make it possible, it was necessary to create mechanisms regulating the work of the digestive tract at the neuronal and endocrine levels [20]. Lymphatic tissue associated with the gastrointestinal tract (GALT) or the enteric nervous system (ENS) is such a mechanisms. The enteric nervous system, which is part of the autonomic nervous system, regulates its work by controlling the muscles, blood vessels or the secretory activity of the mucosal epithelium [21]. It consists of ganglia and nerve fibers lying in the wall of the esophagus, stomach, small and large intestines, pancreas, bile ducts and gallbladder [22]. The change in neurotransmitter coding is influenced by a number of factors, including age [23, 24], intestinal microbiota [25, 26], pathological conditions such as diabetes [27, 28] or inflammatory bowel disease (IBD) [29–31], substances which we take more or less consciously, such as non-steroidal anti-inflammatory drugs [32, 33] and bisphenols [34]. In pigs, which are a model organism in human biomedical research and whose anatomical structure resembles the human anatomical structure in the intestines [35–38] we can distinguish three plexuses: myenteric (MP) and submucous, which is further subdivided into the outer (OSP) and inner (ISP) submucous plexus [39]. By secreting neuronally active substances in these plexuses, the ENS can stimulate or inhibit the work of the digestive tract in response to the current conditions of the microenvironment. The myenteric plexus is mainly responsible for the motility of the digestive tract and the submucous plexus for secretory activity [40]. From the neurotransmitters found in the ENS, six were selected to study their activity in response to microplastics. Each of them performs an important function in the ENS to maintain homeostasis [41, 42]. Cocaine and amphetamine-regulated transcript (CART) regulates intestinal motility (inhibits stomach emptying) and inhibits the secretion of gastric juice, but the exact role in the digestive tract is unknown [43]. Galanin (GAL) is responsible for inhibiting intestinal motility or emptying the stomach, participates in inflammatory processes (exhibiting neuroprotective effects, mediates and participates in interactions between the immune and neurological systems and has antiproliferative and proapoptotic effects) and regulates secretory activity (inhibits the secretion of somatostatin, insulin, glucose and stomach acid) [44–46]. Nitric oxide (NO), an atypical neurotransmitter, is generated by nitric oxide synthase (NOS). There are three types of nitric oxide synthases—neuronal (nNOS), inducible (iNOS), and endothelial (eNOS). Nitric oxide, which is produced by these enzymes, is

responsible for inhibiting intestinal motility and promoting vasodilation [47] and is also involved in inflammatory processes [48]. Neuropeptide substance P (SP), as a representative of tachykinins induced the secretion of water and electrolytes. It has a pro-inflammatory effect by stimulating the production of cytokines such as IL-1β, IL-6, IL-8, and TNF-α [49–51]. The vesicular acetylcholine transporter (VAChT) is considered the main marker of cholinergic neurons [52]. The function of acetylcholine is secretory stimulation and the regulation of blood flow [40, 53]. It also exhibits anti-inflammatory activity [54, 55]. The role of vasoactive intestinal peptide (VIP) in the gastrointestinal tract is to dilate blood vessels, increase intestinal blood flow and stimulate intestinal secretion [56, 57]. Like substance P, it has an immunomodulatory effect [50, 58].

Implications in food chain and research needs

The identification of microplastic particles in samples of animal feed [59], food [60–62] and feces [63–68] is clear evidence of the ongoing exposure of the gastrointestinal tract. From the point of view of initiatives such as Farm to Fork (F2F), the presence of microplastics in the food chain threatens the health and safety of consumers. Even despite the actions taken regarding the sustainability of food systems or the European Green Deal, eliminating the threat related to microplastics seems to be unrealistic at present. Studies on porcine cell cultures have been reported in the available literature reports [69, 70]. However, due to the multitude of biochemical processes and the metabolic complexity of the digestive system, it is necessary to conduct in vivo studies. To date, no such studies have been undertaken and the available reports describe only the occurrence of particles in the lungs of pigs from the natural environment [71]. It should be emphasized that the domestic pig (*Sus scrofa domestica*) is considered one of the better models in human biomedical research, especially of the gastrointestinal tract [35, 37, 38]. The doses used in the studies presented were intended to be overload doses and do not reflect the actual amounts of molecules typically ingested by humans and animals. Although these doses were of a cognitive nature, they may prove to be adequate in the future. The use of such high doses allowed for the definition of polyethylene terephthalate microplastics (PET-MP) and their effects on the neuroplasticity of the enteric nervous system in the duodenum to be studied.

Results

Myenteric plexus

In the myenteric plexus (MP) the low dose significantly reduced only the number of nNOS positive neurons ($p < 0.01$), which was also the most numerous population in the C group. In the remaining cases, the low dose did not change the size of the population ($p > 0.05$). In the case of CART and GAL, there was a significant increase in the population of positive neurons in the HD group compared to the C group ($p < 0.001$). The high dose has the weakest impact on the size of population of VAChT and SP-positive neurons, where the significance was ($p < 0.01$) and ($p < 0.05$), the low dose did not cause statistically significant differences ($p > 0.05$). In neurons positive for nNOS and VIP, a decrease in neuronal population was observed ($p < 0.001$). The exact results are presented in Figs. 1, 2.

Outer submucous plexus

There were several similarities with the MP found in the outer submucous plexus (OSP). Altough nNOS-positive cells also in this plexus constituted the largest population in the control group, microplastic supplementation resulted in a decrease in their activity only in the HD group ($p < 0.001$). The least numerous population were CART-positive neurons. In animals receiving low ($p < 0.05$) and high ($p < 0.001$) doses of microparticles, an increase in the number of CART-positive neurons was observed. The high dose increased the percentage of GAL-positive neurons ($p < 0.001$), with no differences due to the low dose ($p > 0.05$). In the case of SP and VIP, a significant decrease in neuronal activity was observed, regardless of the dose taken. The activity of VAChT was significantly reduced ($p < 0.001$) after high dose supplementation. The exact results are shown in Figs. 3, 4.

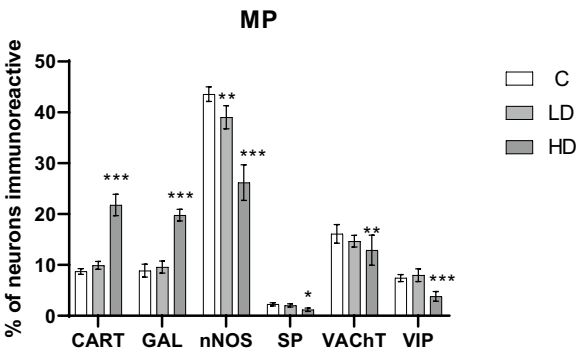


Fig. 1 Percentage of neurons immunoreactive in the myenteric plexus. Percentage (*mean* ± *SEM*) of neurons immunoreactive for CART, GAL, nNOS, SP, VAChT and VIP in control (C) and in animals receiving low (LD) and high dose (HD) of MP-PET. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ indicate statistically significant differences in the expression of the tested substances in relation to the control group

Inner submucous plexus

In the inner submucous plexus, a high dose of MP-PET caused a decrease in SP ($p < 0.001$), nNOS ($p < 0.001$), VAChT ($p < 0.001$) and VIP ($p < 0.001$) positive neurons and an increase in CART ($p < 0.001$) and GAL ($p < 0.001$) positive neurons. The low dose had an effect on the decrease only in the case of nNOS ($p < 0.01$) or VIP ($p < 0.001$) positive neurons, without affecting the number of CART, GAL, SP and VAChT positive neurons ($p > 0.05$). The exact results are shown in Figs. 5, 6. Detailed results of one-way ANOVA for all convolutions are presented in Table 1.

Histological analysis

The structure of the duodenum in the C group was typical for this segment of the pig intestine. The mucosa formed short and irregular villi. Goblet cells were frequently noted in the mucosal epithelium, especially in the crypts. The lamina propria contained numerous lymphocytes and plasma cells. The submucosa was filled by the well-developed Brunner's glands. Pathological changes were not found in the investigated sections of the duodenum of control pigs. In contrast, focal changes with the desquamation of enterocytes and the accumulation of large amounts of mucus were frequently observed in the duodenum sections from the LD and HD groups. The loss of enterocytes occurred both on the apical and lateral surfaces of the villi. The characteristic feature of the villi lacking the epithelium was the presence of blood-containing vessels in their tips. The mucus with cellular debris covered the mucosa forming a thick layer on its surface and frequently filled the spaces between villi. In the affected parts of the intestine, enormous accumulations of goblet cells were noted in the epithelium of the upper parts of crypts and the basal parts of the villi. The lamina propria showed hyperaemia. The pathological changes were more frequently observed in the pigs receiving a high dose of MP-PET (Fig. 7).

A morphometric analysis showed differences in the length of villi, which were significantly lower in the LD and HD groups compared to the control groups and in the pigs receiving a high dose than in those receiving a low dose of MP-PET. The depth of the crypts did not differ between the groups. The thickness of the mucosa was significantly lower in pigs receiving a high dose of MP-PET than in the C group. There were no differences in the thickness of the submucosa and muscularis externa (Table 2).

Ultrastructural study

In group C, the intestinal villi were covered by a continuous epithelial cell layer comprising mainly absorptive cells and goblet cells (Fig. 8A). The apical surface of absorptive cells formed numerous, long, regularly arranged microvilli. The prominent bundles of microfilaments descend from the microvilli into the terminal web. The upper part of the cytoplasm contained numerous mitochondria with moderate electron density of matrix and well-developed cristae. The goblet cells were less frequent than absorptive cells and had a typical structure with an accumulation of large, irregular granules in their widened upper part.

The intestinal villi showed pathological changes in LD (Fig. 8B, C) and HD (Fig. 8D–F) pigs. The extent and character of these changes differed between samples and were correlated with the presence of an enormous, thick layer of mucus on the mucosa surface. In the areas without accumulation of mucous, adsorptive cells showed shortage of microvilli and partial or complete loss of microvilli, although the epithelium still retained its integrity and continuity (Fig. 8C, E). The cells formed variable protrusions (in shape and size) on the apical surface (Fig. 8C, E). As a consequence, the brush border lacked its regular organization. The changes in microvilli correlated with the reduction of the terminal web and changes in the cytoplasm of the absorptive cells. The cells with damaged microvilli frequently comprised mitochondria with an electron-lucent matrix, sometimes obviously swollen (Fig. 8C, E). Some absorptive cells showed vacuolization.

Much more serious damage to the intestine epithelium was observed in the areas of mucosa covered by a thick mucus layer. The severity of the injury enabled three degrees of damage to be distinguished. The first degree included almost a complete loss of microvilli and damage to the apical parts of a few adsorptive cells, but with the remained continuity of the epithelium (Fig. 8B). Numerous goblet cells occurred in these areas. The second degree of injury was defined by the presence of many damaged cells with swollen mitochondria and numerous

(See figure on next page.)

Fig. 2 Myenteric plexus of porcine duodenum. Fluorescence microscope image showing the distribution of neurons immunoreactive to PGP 9.5—used as a pan-neuronal marker and CART, GAL, nNOS, SP, VAChT, VIP in the control group (C) (**A**, **D**, **G**, **J**, **M**, **P**), after administration of low (LD) (**B**, **E**, **H**, **K**, **N**, **R**) and high (HD) (**C**, **F**, **I**, **L**, **O**, **S**) doses of MP-PET. All photographs were made by overlapping green and red fluorescent channels (green for PGP 9.5 and red for CART, GAL, nNOS, SP, VAChT and VIP, respectively). Neurons immuno-positive to a particular substance studied are indicated with arrows

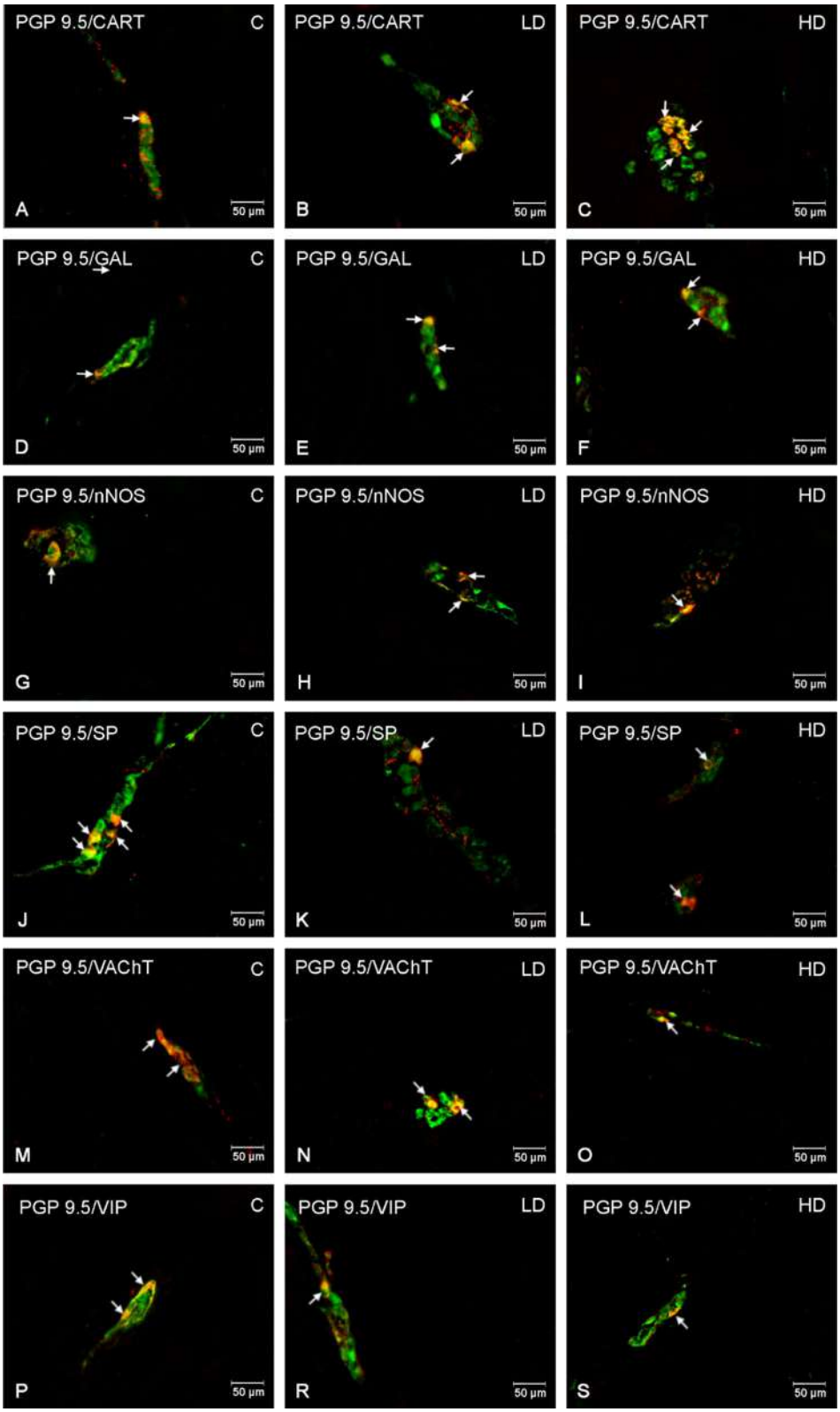


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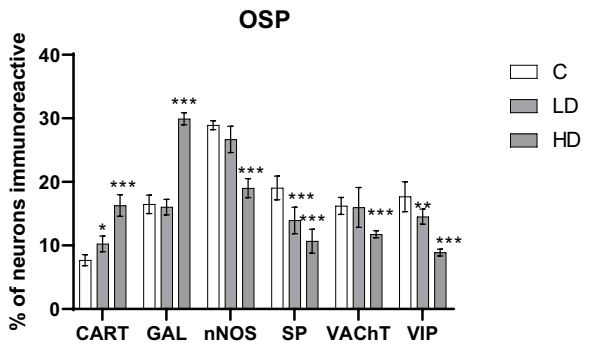


Fig. 3 Percentage of neurons immunoreactive in the outer submucous plexus. Percentage (*mean* ± *SEM*) of neurons immunoreactive for CART, GAL, nNOS, SP, VAChT and VIP in the control (C) and in animals receiving low (LD) and high dose (HD) of MP-PET. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ indicate statistically significant differences in the expression of the tested substances in relation to the control group

vesicles in the cytoplasm (Figs. 8D, F, 9A, B). Spillage of degraded cell remnants into the lumen resulted in breaks in the epithelial continuity. The layer of mucus was extremely thick and contained cellular debris. The third degree included the areas with a complete loss of epithelial cells (Fig. 9C). The connective tissue of the lamina propria was separated from the mucous by the basement membrane, which remained intact in most of the damaged area. The foci without the basement membrane were sporadically found.

The ultrastructure of intestinal glands located beneath the areas with damaged villi, showed no pathological alternations (Fig. 10).

Discussion

Based on the available literature, it can be concluded that the presented research shows for the first time the effect of a cumulative dose of microplastic on the enteric nervous system in a domestic pig. To date, studies have been conducted mainly on mice, rats or aquatic organisms [17, 72–74]. Based on the available literature, the generalized conclusion can be drawn that the effect of microplastics on animals is influenced by several factors. It has been reported that microplastics in vertebrates can accumulate in internal organs such as the ovaries, testes [72, 75], intestines, kidneys, liver [76–79] and lungs [71, 76]. In humans, microplastics have been identified in the liver [80], intestines [81] lungs [82], placenta [83, 84] milk [85] and sputum [86]. Studies conducted by Donkers et al. [87] on advanced in vitro models representing the lungs and intestines using various types of micro- and nano-plastics, aimed at demonstrating the potential of these molecules to induce cytotoxic and pro-inflammatory effects. They confirmed the ability to translocate plastic particles through the lung and intestinal epithelium, as well as the ability to activate pro-inflammatory cells [87]. The ability to penetrate the intestinal barrier suggests that orally ingested microplastics may spread throughout the body through the bloodstream and accumulate in tissues. This phenomenon can be described as plasticemia—the presence of microplastic particles in the peripheral blood. However, the accumulation of microparticles can lead to organ dysfunction and impairment of one system or even the whole organism.

Senathirajah et al. predicted that the standard consumer is exposed to 0.1–5 g/week of microplastic (approximately equivalent to the weight of a credit card) [88]. Pletz claims that these data are overestimates [89]. Cox et al. [14] evaluate the annual consumption at the level of 39,000 to 52,000 particles depending on age and gender. Mohamed et al. conducted a probabilistic assessment trying to estimate how many particles will be ingested by a standard consumer over a lifetime and reported 553 particles/capita/day for children and 883 particles/capita/day for adults) [90]. A large discrepancy can be observed in the mentioned research. This may result from only hypothetical assumptions, burdened with error and high variance and even socioeconomic status. This state of knowledge confirms that it is impossible to clearly determine what effect microplastics have on living organisms, because it depends on too many variables, such as the shape, size or number of particles, and without knowing the amount of ingested particles it is impossible to determine how it affects the body and, most importantly, how to effectively counteract these effects. In addition, as it is not a biological substance, standard analytical methodology is difficult to apply. Larger plastic fragments can cause physical tissue damage, such as the lateral line of fish [91], and smaller ones can penetrate deep into the body, e.g. to the intestines [92]. On the other hand, there are reports that microplastics do not have a significant effect on fish development as was supposed [93, 94]. Undoubtedly, microplastics and

(See figure on next page.)

Fig. 4 Outer submucous plexus of the porcine duodenum. Fluorescence microscope image showing the distribution of neurons immunoreactive to PGP 9.5 —used as a pan-neuronal marker and CART, GAL, nNOS, SP, VAChT, VIP in the control group (C) (**A**, **D**, **G**, **J**, **M**, **P**), after administration of low (LD) (**B**, **E**, **H**, **K**, **N**, **R**) and high (HD) (**C**, **F**, **I**, **L**, **O**, **S**) doses of MP-PET. All photographs were made by overlapping green and red fluorescent channels (green for PGP 9.5 and red for CART, GAL, nNOS, SP, VAChT, and VIP, respectively). Neurons immuno-positive to a particular substance studied are indicated with arrows

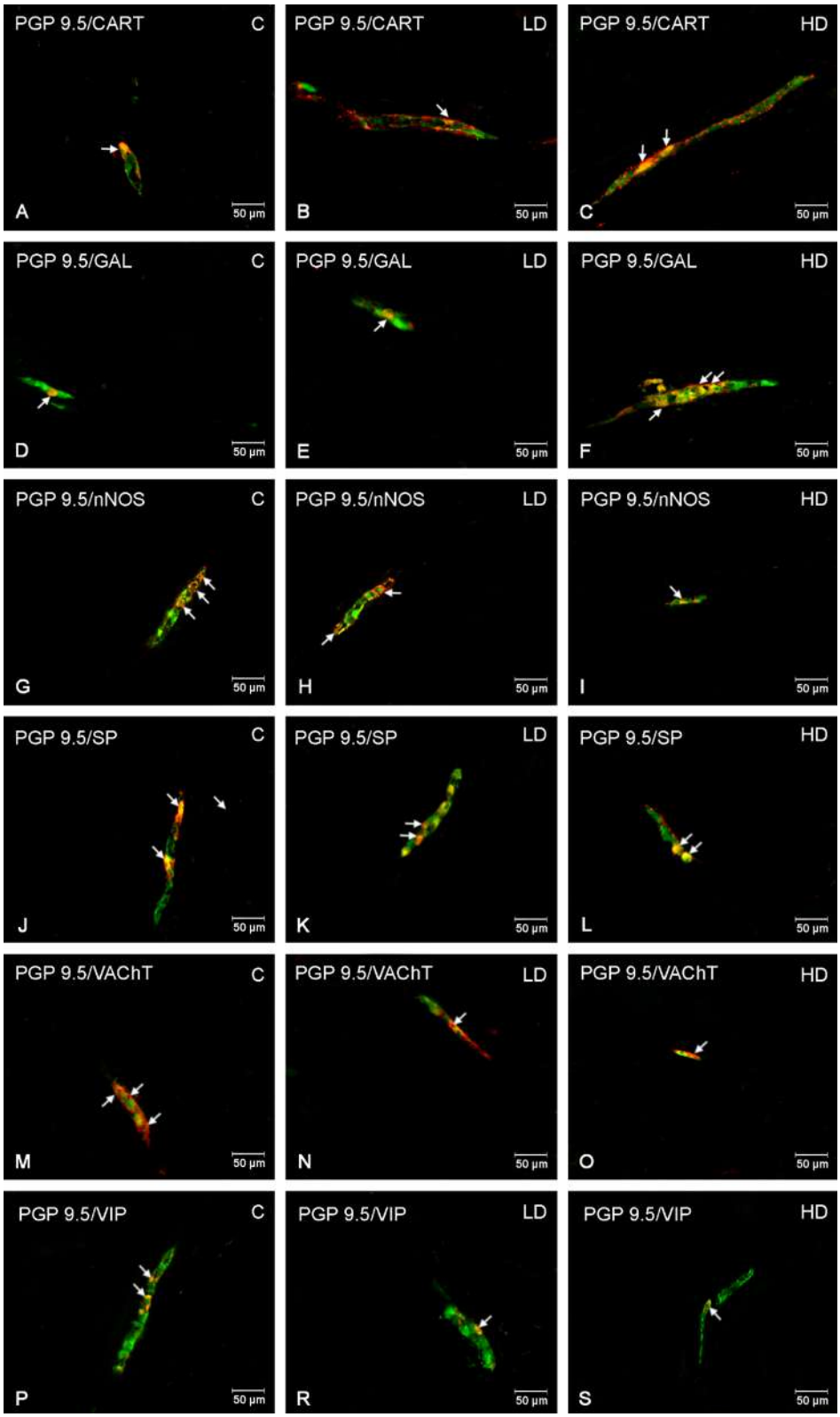


Fig. 4 (See legend on previous page.)

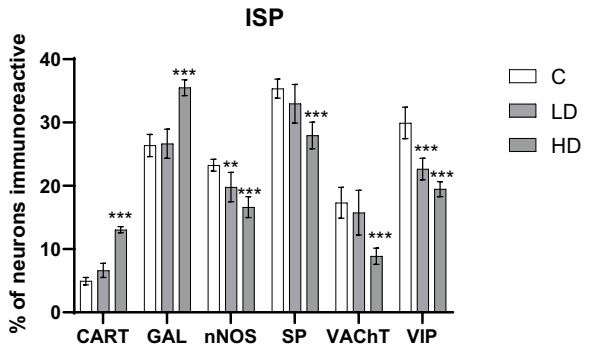


Fig. 5 Percentage of neurons immunoreactive in the inner submucous. Percentage (*mean* ± *SEM*) of neurons immunoreactive for CART, GAL, nNOS, SP, VAChT and VIP in the control (C) and in animals receiving low (LD) and high dose (HD) of MP-PET. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ indicate statistically significant differences in the expression of the tested substances in relation to the control group

the correlated environmental burden are part of a One Health issue [95, 96], and thus requires a multidisciplinary approach to manage the risks posed to humans, animals and the environment.

Previous studies have shown that the enteric nervous system, due to its plasticity, adapts to the influence of adverse factors such as chemicals or inflammation of the intestines and diabetes [32, 33, 97–100]. This is possible due to a dense network of neurons in the intestines, which form plexuses and depending on the conditions, modify the secretion of neuronally active substances. The proper functioning of the enteric nervous system affects not only the digestive system, but also the entire body. In the case of neurodegenerative diseases such as Parkinson's disease, dysfunction in the functioning of the ENS has been observed, and is suspected as the potential origin of this disease [101]. The results presented in this article suggest that the occurrence of microplastics may be correlated with inflammation in the intestine [102]. The confirmation of the above may be a positive correlation between the concentration of microplastics in the feces and the severity of IBD symptoms [102]. Currently, however, it is not possible to determine whether the presence of IBD increases the retention of microplastics in the intestines or whether microplastics increase inflammatory symptoms [102].

The presented results confirm the adaptation of the enteric nervous system in response to PET-MP doses expressed by changes in the population of neurons CART, GAL, nNOS, SP, VAChT and VIP positive. The severity of changes varied depending on the examined plexus. In the duodenum, food passed from the stomach is mixed with bile from the gallbladder and pancreatic juice. This allows the neutralization of stomach acid and the absorption of nutrients, vitamins and minerals. Changes in chemical coding in this section of the digestive tract may affect the absorption of substances in further sections of the intestine. The digestive process initiated in the stomach changes the surface of plastics, generating fine particles on their surface [103]. A molecule that passes through the digestive tract may show different morphological features (changes in size, shape and chemical properties). This may result in a different effect on the ENS depending on the examined part of the gastrointestinal tract. In order to confirm this thesis, it is suggested to perform studies determining the population of positive neurons in various sections of the digestive tract, as well as to determine the exact changes that microplastic undergoes during the in vivo digestion process.

Cocaine and amphetamine-regulated transcript have a gastric acid-blocking effect [43]. It has neuroprotective properties [104]. Under the influence of bisphenol A, a decrease in the population of CART-positive neurons was observed in the duodenum [34], unlike indomethacin [33], acrylamide [105] or glyphosate [99]. The significant increase in CART neurons in the present study can be explained by the neuroprotective effect. Inhibiting the secretion of gastric acid may reduce the exposure of microplastics to digestive enzymes, and therefore less likely to cause changes in the ingested molecules and altered interaction in further sections of the digestive tract.

Vasoactive intestinal peptide induces relaxation of smooth muscles and stimulates the secretion of water, electrolytes and secretion of bicarbonates by the pancreas. It is responsible, like CART, for the inhibition of gastric acid secretion. Through these actions it increases intestinal motility [56, 106]. Vasoactive intestinal peptide has a neuroprotective effect through glial cells that produce cytokines. The decrease in VIP-positive neurons noted in the present study was also observed with exposure to bisphenol A [34] and lipopolysaccharides [107]. However, most inflammatory conditions result in an increase in VIP

(See figure on next page.)

Fig. 6 Inner submucous plexus of porcine duodenum. Fluorescence microscope image showing the distribution of neurons immunoreactive to PGP 9.5 —used as a pan-neuronal marker and CART, GAL, nNOS, SP, VAChT, VIP in the control group (C) (**A**, **D**, **G**, **J**, **M**, **P**) after administration of low (LD) (**B**, **E**, **H**, **K**, **N**, **R**) and high (HD) (**C**, **F**, **I**, **L**, **O**, **S**) doses of MP-PET. All photographs were made by overlapping green and red fluorescent channels (green for PGP 9.5 and red for CART, GAL, nNOS, SP, VAChT, VIP respectively). Neurons immuno-positive to a particular substance studied are indicated with arrows

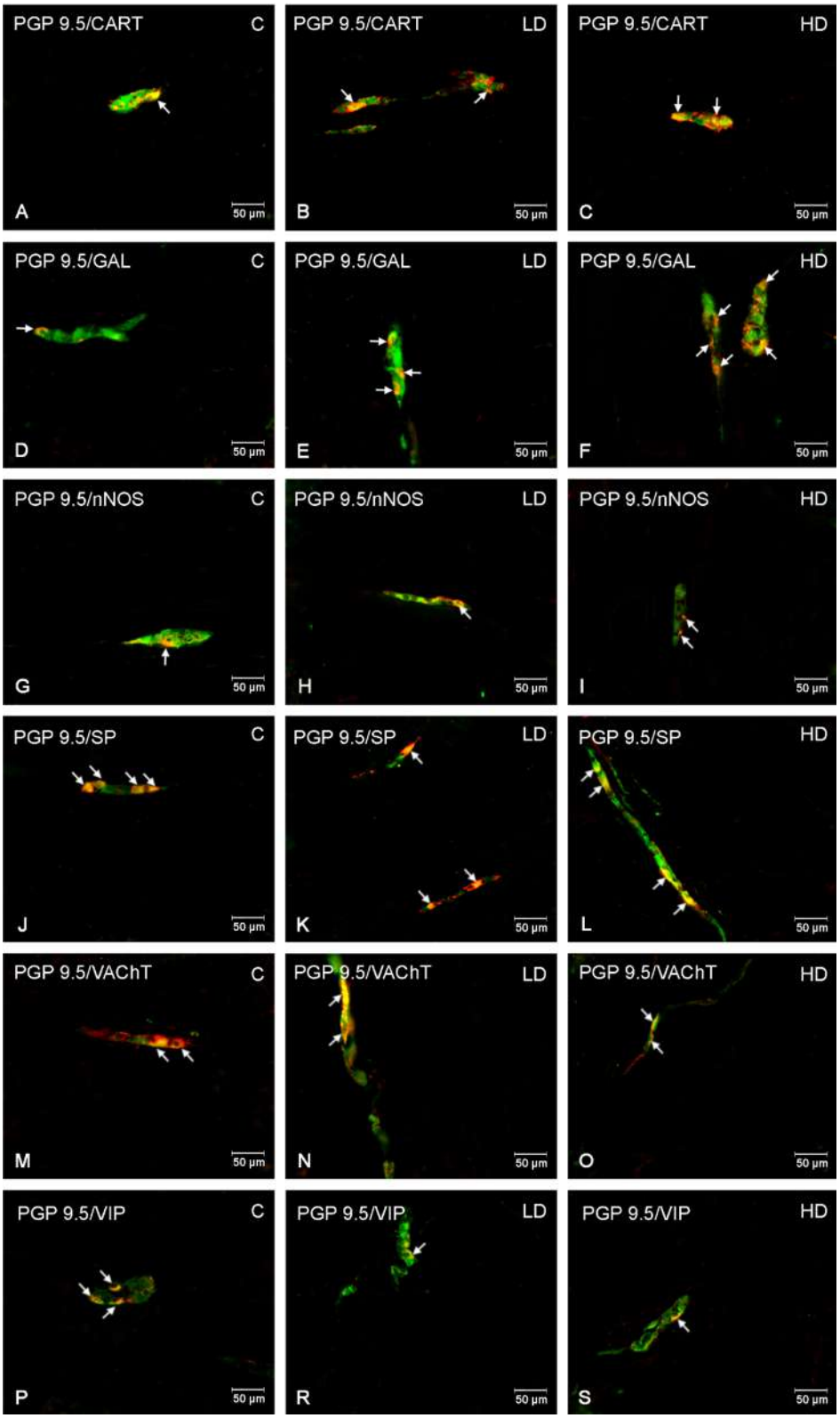


Fig. 6 (See legend on previous page.)

Table 1 Detailed results of one-way analysis of variance

	For the model				For research groups			
	df	*MS*	*f*	*p* Value *	*df*	*MS*	*f*	*p* Value *
MP								
CART	1	145.03	1300.21	< 0.001	2	13.55	121.48	< 0.001
GAL		155.47	1489.21	< 0.001		12.07	115.62	< 0.001
nNOS		1016.88	4503.16	< 0.001		20.75	91.89	< 0.001
SP		2.80	154.76	< 0.001		0.09	5.02	< 0.01
VAChT		163.56	1318.69	< 0.001		0.65	5.25	< 0.01
VIP		31.50	530.01	< 0.001		1.30	21.85	< 0.001
OSP								
CART	1	98.09	983.90	< 0.001	2	4.90	49.19	< 0.001
GAL		342.78	2140.04	< 0.001		16.16	100.88	< 0.001
nNOS		478.51	2584.45	< 0.001		7.01	37.87	< 0.001
SP		164.71	1328.36	< 0.001		4.55	36.71	< 0.001
VAChT		164.42	1324.79	< 0.001		1.68	13.53	< 0.001
VIP		142.01	1215.64	< 0.001		5.00	42.77	< 0.001
ISP								
CART	1	50.75	685.37	< 0.001	2	4.61	62.30	< 0.001
GAL		710.16	3454.10	< 0.001		7.26	35.34	< 0.001
nNOS		308.74	1951.41	< 0.001		2.97	18.79	< 0.001
SP		803.64	3710.00	< 0.001		3.80	17.55	< 0.001
VAChT		148.91	1249.38	< 0.001		5.14	43.11	< 0.001
VIP		439.24	2429.47	< 0.001		7.26	40.15	< 0.001

MP—myenteric plexus, OSP—outer submucous plexus, ISP—inner submucous plexus, CART—cocaine and amphetamine regulated transcript, GAL—galanin, nNOS—neuronal nitric oxide synthase, SP—substance P, VAChT—vesicular acetylcholine transporter, VIP—vasoactive intestinal peptide, *df*—degrees of freedom, *MS*—mean squares, *f*—Fisher F ratio, * statistical significance at $p < 0.05$.

activity due to its immunomodulatory and neuroprotective properties [108]. In the present studies, PET-MP reduced the activity of VIP in the duodenum, which may have a neurodegenerative effect on the neurons of the examined segment of the intestine.

Galanin exhibits neuroprotective properties and acts as an inhibitory neuromodulator [46]. An increase in GAL activity has been noted in pigs after long-term supplementation with a non-steroidal anti-inflammatory drug [33] acrylamide [109] or in dogs with IBD [31]. Galanin may affect the motility of the gastrointestinal tract by inhibiting acetylcholine and SP via excitatory GAL-R_1 receptors [110]. In the present results, the population of GAL-positive neurons increases and the population of VAChT and SP-positive neurons decreases. This confirms the assumptions of the inhibitory and anti-inflammatory effects of GAL in the ENS. Reduced intestinal motility may result in the retention of intestinal contents containing microplastics, which may promote the entry into the bloodstream of molecules able to overcome the intestinal barrier.

The vesicular acetylcholine transporter (VAChT) is considered the main marker of cholinergic neurons [52]. The function of acetylcholine is secretory stimulation and the regulation of blood flow [40, 53]. It also exhibits anti-inflammatory activity by lowering the level of pro-inflammatory cytokines [54, 55]. A decrease in the population of VAChT-positive neurons is observed in the case of exposure to glyphosate [99] or bisphenol A [34], while an increase is observed after supplementation with acrylamide [109] or in mice with Huntington's disease [111]. Bisphenol A is one of the components that is used in the production of plastics, and it causes a similar effect as the tested PET-MP. A reduced level of acetylcholine may promote the development of inflammation in the intestines.

The activity of SP is mainly correlated with the occurrence of inflammation [112] and is associated with pain symptoms [113]. It stimulates the production of pro-inflammatory cytokines through the neurokinin-1 receptor (NK_1R) [51], and regulates the transport of ions and fluids through the epithelium to the intestinal lumen [114]. A decrease in SP activity was noted with long-term exposure of pigs to naproxen [32] and in rats after administration of ulcerogenic agents [115], with multiple organ dysfunction syndrome [116] and diabetes [117, 118]. This has been associated with the relaxation of the intestinal

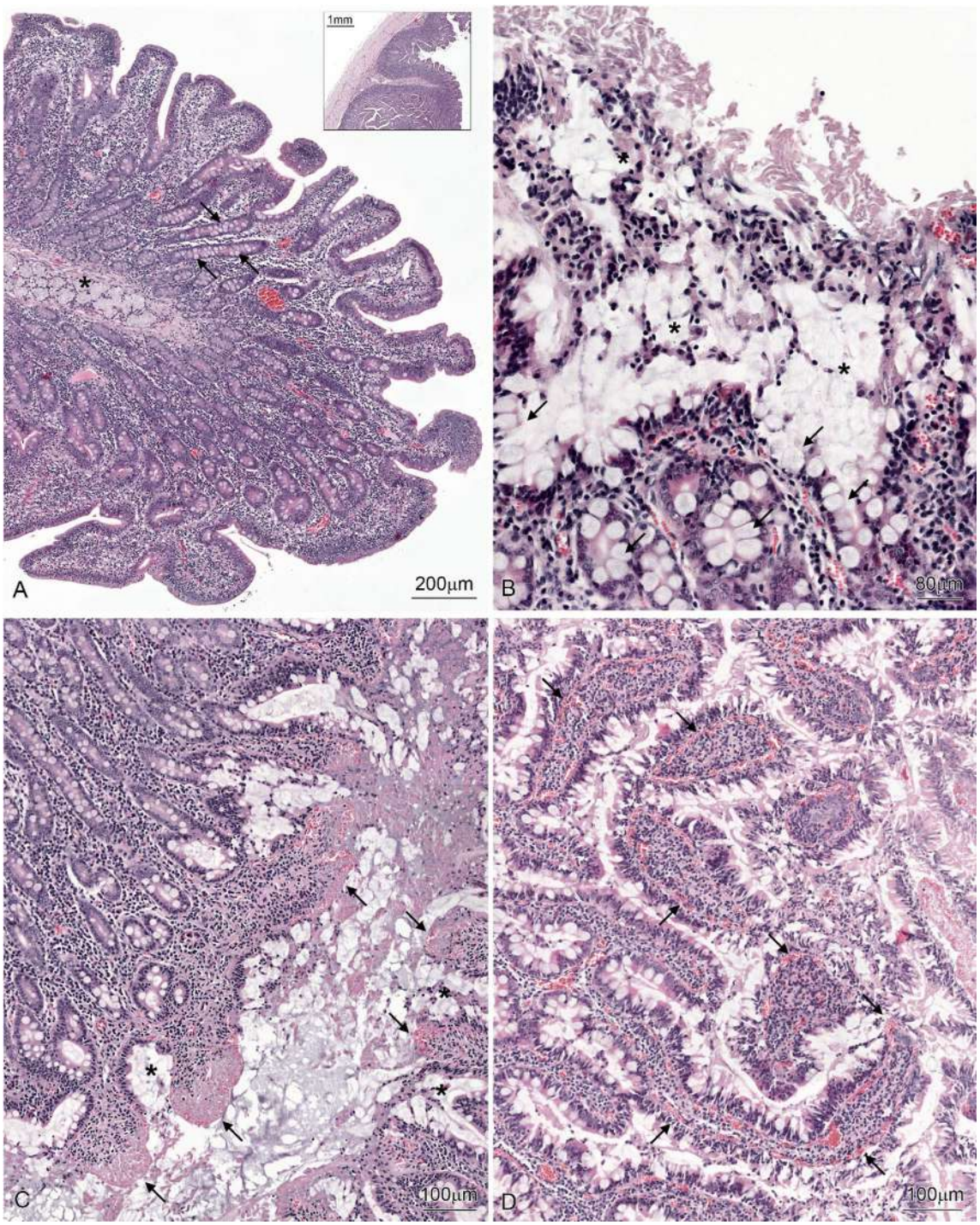


Fig. 7 Histological structure of the duodenum in a C, LD and HD group. **A** Note short and irregular villi, numerous goblet cells (arrows) in the crypts, and the Brunner's glands (asterisk). The insert shows the section though the entire wall of the duodenum. **B** Note presence of numerous goblet cells (arrows) in the epithelium and the thick mucus layer containing cellular debris (asterisks). **C** Note the detachment of enterocytes from the villi (arrows) and the presence of mucus with cellular debris between the villi (asterisks). **D** Note numerous blood-containing vessels (arrows)

muscle leading to motility disorders or with the formation of ulcers in the duodenum. In humans with constipation [119] or with diabetes, a decrease in SP levels has been observed in the rectal [120] and gastric [121] mucosa. An increase in SP activity can be observed much more often under the influence of various factors causing

Table 2 Statistical analysis of histological study

Parametr (µm)	C	LD	HD
Length of villi	356.38 ± 46.57	315.81*** ± 45.14	272.14*** ± 45.22
Deep of crypts	594.25 ± 85.62	585.87 ± 68.27	561.37 ± 98.34
Thickness of mucosa	915.97 ± 115.79	920.00 ± 113.66	779.90*** ± 114.14
Thickness of submucosa	232.58 ± 44.99	219.94 ± 44.77	221.64 ± 49.79
Thickness of muscularis externa	423.05 ± 54.44	411.20 ± 63.64	430.5*** ± 56.14

The length of villi, the deep of crypts, the thickness of mucosa, the thickness of submucosa, and the thickness of the muscularis externa in the duodenum of pigs from the C, LD and HD group (*mean* ± *SD*). ***$p < 0.001$ indicate statistically significant differences in the expression of the tested substances in relation to the control group.

inflammation in the intestines [33, 34, 109]. The decrease in the number of positive SP in the results presented in the article may result from the degree of inflammation, degeneration or necrosis of neurons under the influence of a toxic agent [106].

The variability in the number of nNOS-positive neurons is an occurrence often observed in gastrointestinal disorders. Nitric oxide is considered one of the main inhibitory factors, causing the relaxation of the intestinal muscles, the inhibition of the secretion of intestinal hormones and the regulation of the transport of water and electrolytes. Because nNOS inhibit the secretion of gastric juice, it has a protective function [122]. Diabetes in pigs [28] and exposure to acetylsalicylic acid [123] causes a decrease in the number of nNOS-positive neurons, while an increase in the population of nitrergic neurons is observed in dogs with IBD [100] and diabetic rats [124]. In diseases where the intestinal mucosa is only slightly damaged, elevated levels of NO are reported [122]. Polyethylene induces NO production in human cell lines with pro-inflammatory properties [125]. Whether NO has pro- or anti-inflammatory properties is not fully understood. The observed decrease in the level of nitric oxide under the influence of PET-MP may adversely affect intestinal neurons.

A decrease in nNOS, VAChT and VIP-positive neurons may be one of the defense mechanisms against microplastics. These neurotransmitters can cause vasodilation, which in the case of inhibition of intestinal motility caused by (among others) the activity of galanin, creates conditions conducive to the absorption of microparticles from the intestines into the bloodstream. Primarily, acetylcholine and SP-secreting neurons are excitatory neurons. They stimulate smooth muscles, increase the secretion of intestinal juice, dilate blood vessels and secrete intestinal hormones [110]. PET-MP in the current study reduced the population of the previously mentioned neurons. This may result in a negative effect on intestinal peristalsis (affecting the MP) and digestive functions (affecting the OSP and ISP). Reducing the activity of SP and VIP may weaken the immune response by inhibiting the activity of macrophages [107]. The presented results correspond with the observation obtained by Szymanska et al. [34] on bisphenol A. Similar population behaviors were noted in the population of VIP and VAChT positive neurons, but different in the SP and GAL populations. Bisphenols are not used in the production of PET packaging, although, other agents used in the production of PET products may act in a similar way. It should be noted that each plastic differs in chemical composition, which may cause convergent or divergent effects on the body. It is worth noting that exposure to microplastics outside of controlled laboratory conditions varies greatly. The mixture of molecules taken orally is not uniform in composition or size. This fact may generate various effects on the enteric nervous system which are different from the results obtained in the current study.

The analysis of histological changes suggests a negative effect caused by the mechanical impact of MP-PET particles on the duodenal mucosa. The diverse shape of MP-PET, including the presence of particles with potentially sharp edges (Fig. 11), increases the likelihood of cell damage, including focal desquamation of enterocytes. Mucus present in the intestines has a protective function and prevents the adhesion of microorganisms [126]. Mice exposed to oral polypropylene showed a decrease in mucus production and the destruction of the intestinal mucosal barrier in response to oxidative damage and altered inflammatory cytokine levels [127]. It can be assumed that MP-PET initially caused the loss of microvilli, to which the body responded with increased mucus secretion as a defense mechanism. In the duodenum of the mouse [128] similarly to the presented study, the proliferation of small blood vessels and infiltration of inflammatory cells such as lymphocytes and plasma cells in the lamina propria were found. The influence of microplastics on the cells of the digestive tract in *Daphnia magna* [129] and *Artemia parthenogenetica* [130] corresponds to the results obtained, i.e. the shortening of the length of the microvilli and their damage. In smaller animals such as *Dapnia* and *Artemia* this may result in stunted growth or increased mortality. In pigs, these effects have not been observed, probably due to a significantly larger area

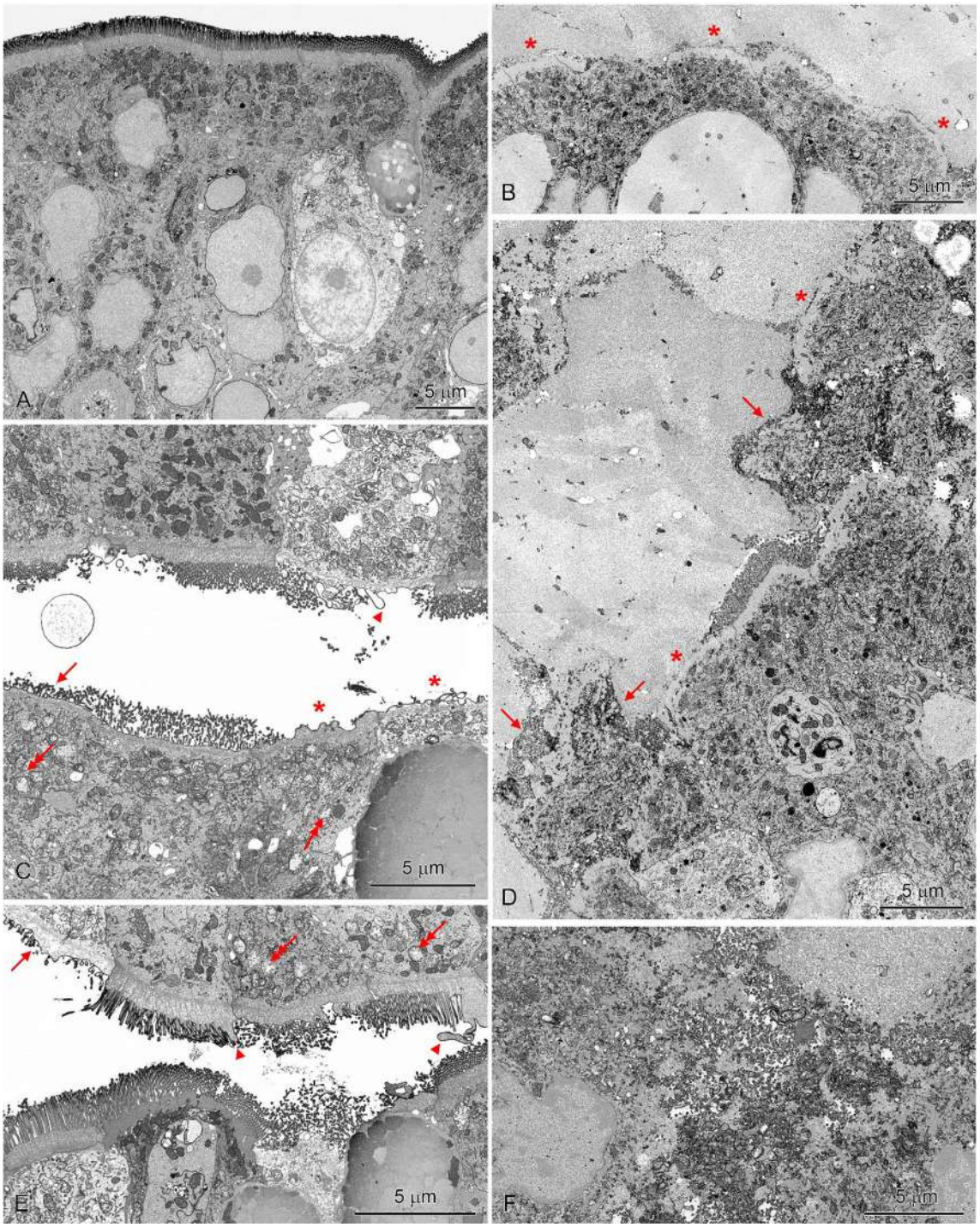


Fig. 8 Ultrastructure of the duodenum epithelium in a C group (A), LD (B, C) and HD group (D, E, F). **A** Note numerous, long, regularly arranged microvilli on the apical surface and numerous mitochondria with a moderate electron density of the matrix in the upper part of absorptive cells. **C**, **E** Note shortage of microvilli (arrows), loss of microvilli (asterisks), protrusions on the apical surface of endothelial cells (arrow heads) and swollen mitochondria (double arrows). **B**, **D**. Epithelium covered by a thick layer of mucus. A lack of microvilli (asterisks) and damage of epithelial cells (arrows). F. Damaged cells in the area covered by a thick mucus layer

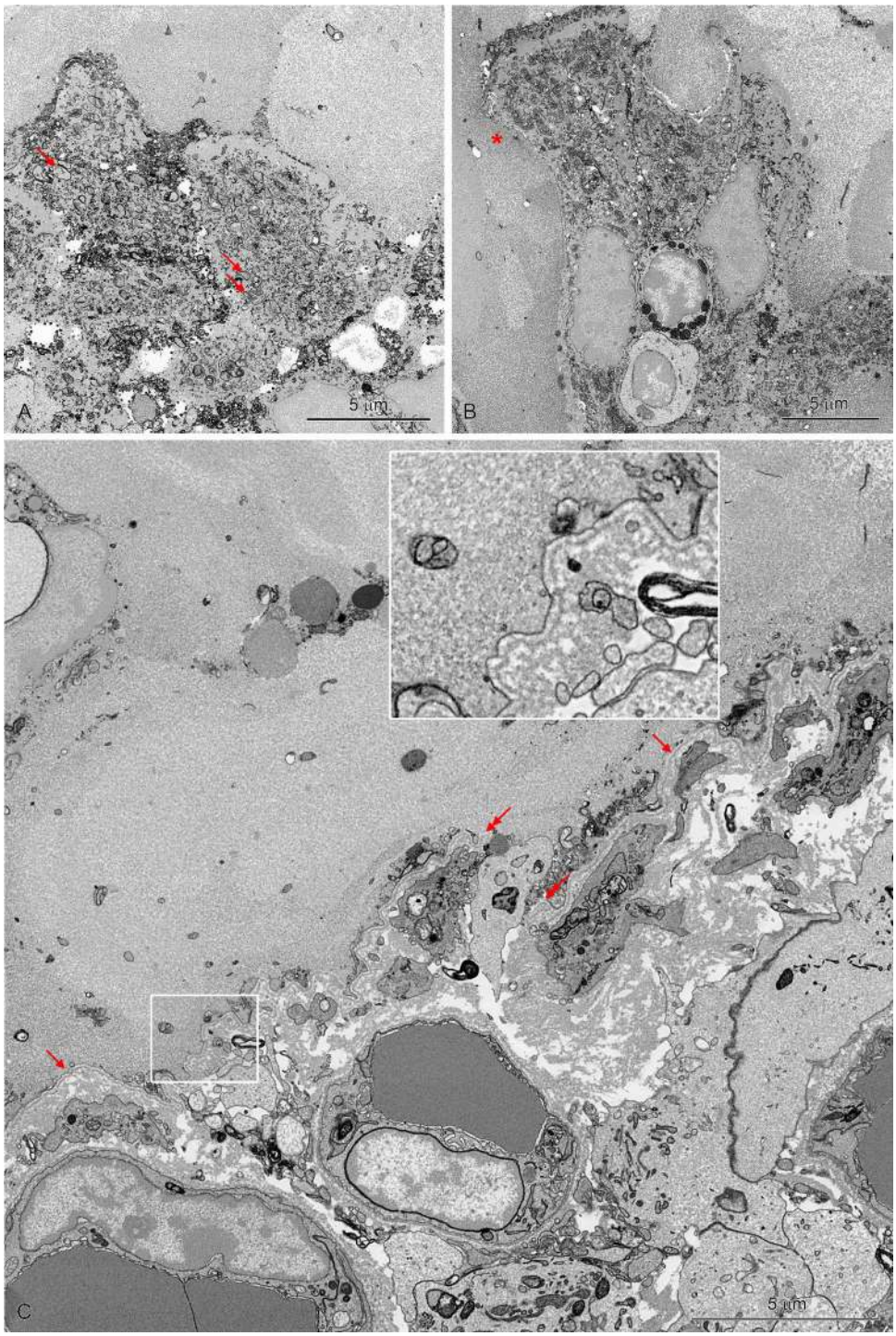


Fig. 9 Ultrastructure of the duodenum epithelium covered a thick mucus layer in the HD group. **A** Note swollen mitochondria (arrows) and numerous vesicles in the cytoplasm of damaged cells. **B** A lack of epithelium continuity (asterisk). **C** Area with a complete loss of epithelial cells. Note that the lamina propria is separated from the mucous by the basement membrane (arrows, insert—a part of the image at higher magnification). Double arrows show a break in the basement membrane

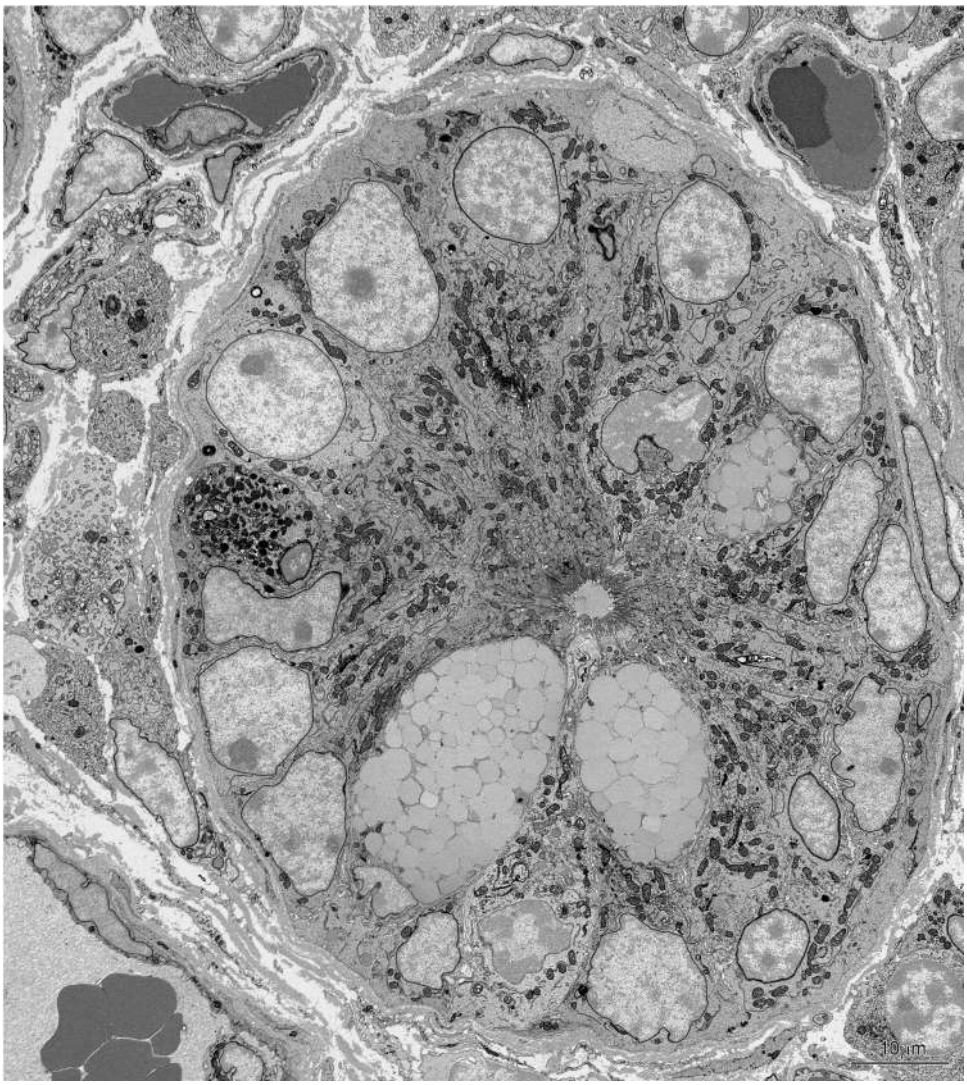


Fig. 10 Ultrastructure of intestinal glands located beneath the area with damaged villi in the HD group. Note the normal organization of cells

of the gastrointestinal tract and the selected dose was too low to cause such an effect.

Conclusion

Based on this study, it may be assumed, that oral intake of microplastic might have a potential negative influence on the digestive tract. The changes observed in the chemical coding of the enteric nervous system in the duodenum, along with the structural changes, confirm that the body is taking action to counteract the inflammation caused by the microplastic. Changes are more pronounced in the group of animals receiving microplastics at a dose of 1 g/day than in the group receiving 0.1 g/day. Despite not having precise knowledge about the amount of microplastics taken in by humans, the state that may be dealt with in connection with the widespread use of plastics is illustrated by the current study.

Material and methods

The aim of this study was to evaluate, how two different doses of polyethylene terephthalate PET (the material most often chosen for packaging beverages and water), affect the enteric nervous system and the morphology of swine duodenum.

Animals and experimental procedures

The experiment was carried out on 15 sexually immature Pietrain X Duroc breed gilts, approximately eight weeks old, weighing approximately 20 kg, from a farm in Lubawa (Poland). The start of the study was preceded

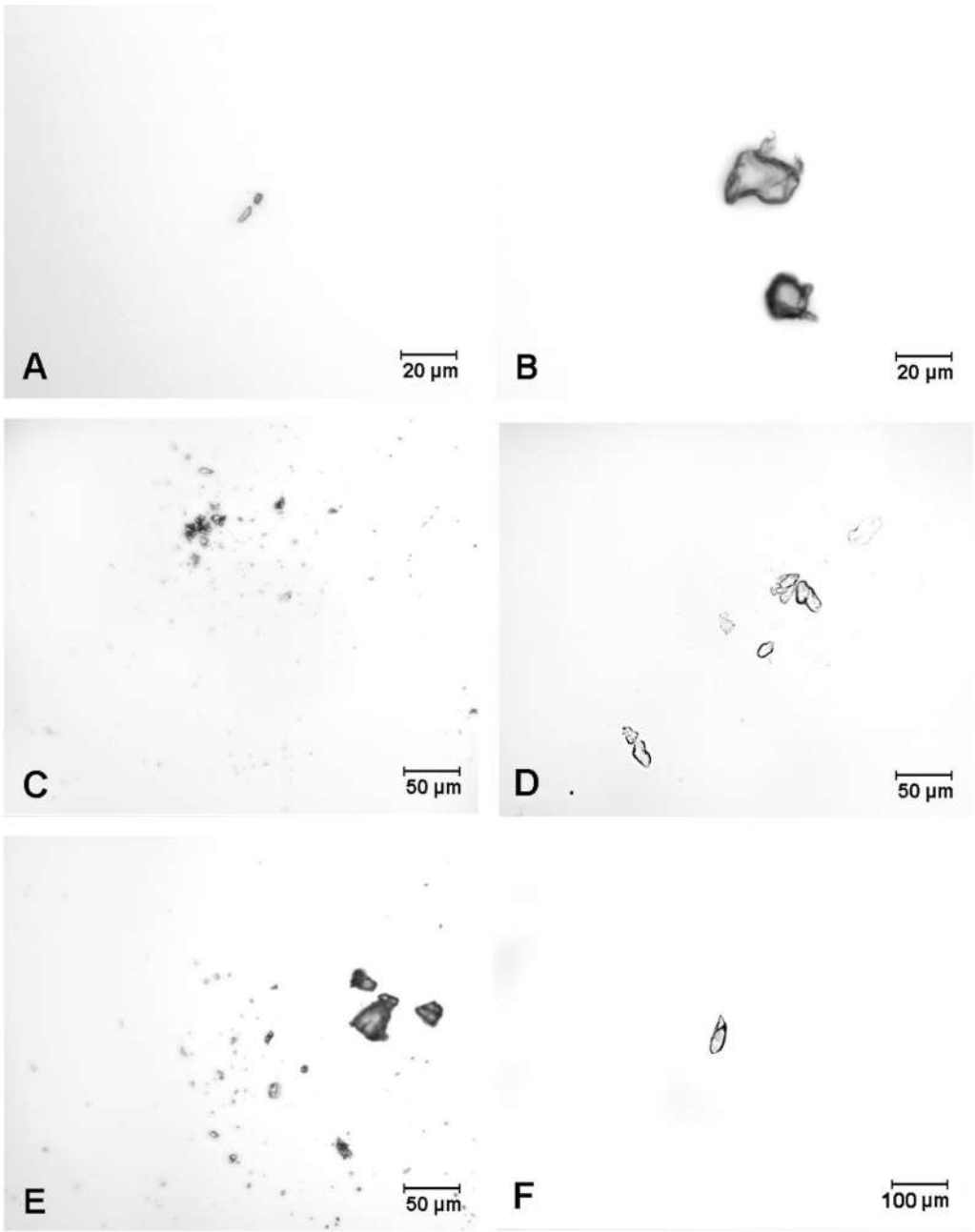


Fig. 11 Particles of polyethylene terephthalate used in the experiment

by obtaining consent from the Local Ethics Committee (decision no. 10/2020 of 26 February 2020). All procedures were carried out in accordance with Polish law, which defines the conditions and methods of conducting experiments on animals and the European Community Directive on the ethical use of experimental animals. The animals were kept under standard laboratory conditions (20–22°C, 55–60% relative humidity, 12 h/12 h light-night cycle) in the animal rooms of the Faculty of Veterinary Medicine of the University of Warmia and Mazury in Olsztyn, Poland. The animals were fed twice a day with commercial feed, with unlimited access to water. All plastic items have been removed from the animals environment. The animals were randomly divided into three study groups (n = 5/group). The control group (C) received empty gelatin capsules once a day for 28 days. The first research groups (LD and HD) received daily gelatin capsules with PET particles (cat. no. ES306031/1,

Good Fellow) (Fig. 11) as a mixture of particles of various sizes (spherical, fibrous, irregular). For particle analysis, 500 randomly selected particles were subjected to microscopic analysis with the use of Zeiss Axio Imager.M2 fluorescence microscope (Zeiss, Oberkochen, Germany) and software ZEISS ZEN Microscopy Software (Zeiss, Oberkochen, Germany). Randomization was performed by obtaining a representative sample of the entire material from different depth levels and then splitting it into 25 microscope slides. Then, 20 randomly selected particles were measured on each slide. The average length of the larger side was 153.09 µm, *Min* – 1.25 µm, *Max* – 299.75 µm, *SD* – 85.13 and *SEM* – 3.81. The first study group (LD) received at a dose of 0.1 g/animal/day and the second study group (HD) received a dose ten times higher—1 g/animal/day. The low and high dose of microplastics were developed on the basis of the logarithm of the decimal (for $\log(0,1) = -1$; for $\log(1) = 0$). The capsules were administered one hour before the morning feeding. The animals were euthanized after a period of 28 days. Premedication was performed using Atropine (0.05 mg/kg *i.m.*, Polfa, Warsaw, Poland), followed by induction with xylazine (3 mg/kg *i.m.*, Vet-Agro, Lublin, Poland) and ketamine (6 mg/kg *i.m.,* Vetoquinol Biowet, Gorzów Wlkp., Poland). After approximately 20 min, an overdose of sodium pentobarbital (0.6 ml/kg, *iv.*, Biowet, Puławy, Poland) was administered. After confirming that life functions had stopped (lack of pupillary reflex, pulse and respirations), the material was immediately collected for further examination. About 4 cm of duodenal fragments (approx. 10 cm away from the pyloric sphincter muscle) were collected for immunofluorescence and histopathological examination.

Double immunofluorescent staining

To fix the preparations, duodenal fragments were placed in a 4% paraformaldehyde solution (pH 7.4) for one hour, then rinsed with 0.1 M phosphate buffer (pH 7.4, every 24 h, 3 times) and finally transferred to 18% sucrose solution (pH 7.4) for two weeks at 4 °C. The sucrose solution was replaced every 10 days. The duodenal fragments were frozen using a Tissue-Tek O.C. T. (Sakura Finerek USA, Inc., Torrance, CA, USA) and a cryostat (CM 1860, Leica, Germany), cut perpendicularly to the intestinal lumen into 14 µm fragments and affixed to chrome-alum-coated microscope slides. Tissues prepared using this method were kept at -20 °C until staining was performed. After drying the sections at room temperature for 45 min and washing them three times in 0.1 M phosphate-buffered saline (PBS, pH 7.4 for 15 min) samples were blocked with the blocking mixture consisting of 10% horse serum and 0.1% bovine serum albumin in 0.1 M PBS, 1% Triton X-100, 0.05% Thimerosal, and 0.01% sodium azide in room conditions for an hour. The next step involved washing three times with PBS solution. Duodenal sections were covered overnight with a mix of primary antisera PGP 9.5 (panneuronal marker) and CART, GAL, nNOS, SP, VAChT or VIP, at room temperature in a water bath. After 24 h, after three washes with PBS, the sections were incubated for 1 h with Alexa Fluor 488 and Alexa Fluor 546 secondary antibodies. At the end of the procedure, the sections were secured with carbonate-buffered glycerol (pH 8.6) and coverslipped. The list of antibodies used in the procedure along with the concentrations used is provided in the Table 3. Stained sections were analyzed under a Zeiss Axio Imager.M2 fluorescence microscope (Zeiss, Oberkochen, Germany) equipped with the appropriate set of filters. A camera connected to a PC with ZEISS ZEN Microscopy Software (Zeiss, Oberkochen, Germany) was used for image acquisition. To determine the percentage of neurons immunoreactive to selected neuroactive substances, at least 500 PGP 9.5 positive neurons (with a clearly visible nucleus) were counted in each duodenal plexus, which were counted as 100% of the population. Then the filter was changed to visualize CART, GAL, nNOS, SP, VAChT or VIP positive neurons and the percentage population of the selected ones was determined. To avoid double counting of the same neurons where a mixture of the same antibodies was used, the distance between the sections was at least 200 µm.

Histological study

The tissue was fixed in 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4) for 48 h. Next, it was dehydrated anethanol (50%, 70%, 90%, 96%, 96%, 99.9%), ethanol 99.9%-xylene mixture (1:1), xylene (3 times) and paraffin (2 times) series (TP 1020, Leica, Germany). The whole process took 23 h. After that, the samples were embedded in paraffin (EG1150, Leica, Germany). The 4 µm-thick sections were prepared using microtome (HM 340E, Microm, Spain) and stained with hematoxylin and eosin method (HE) using a multi-stainer (ST5020 + CV5030, Leica, Germany). The slides were digitalized in Pannoramic 250 Flash scanner (3DHistech, Budapest, Hungary). Morphometric analyses were performed manually using SlideViewer 2.6 software (3DHistech, Budapest, Hungary) and included the length of villi, the crypt deep, the thickness of mucosa, the thickness of submucosa, and the thickness of the muscularis externa. The measurements were performed on three sections per animal, separated from each other by at least 1 cm, in 10 replicates per slide, and the mean values were subjected to a statistical analysis.

Table 3 Antibodies used in the immunofluorescence method

Antigen	Species	Dilution	Code	Supplier
PGP 9.5	Mouse	1:1000	480012	ThermoFisher Scientific, Waltham, MA, USA
CART	Rabbit	1:16,000	H-003-61	Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA
Gal	Guinea pig	1:2000	T-5036	Peninsula, San Carlos, CA, USA
nNOS	Rabbit	1:3000	PA1-033	ThermoFisher Scientific, Waltham, MA, USA
SP	Rat	1:150	8450-0505	BioRad, Hercules, CA, USA
VAChT	Rabbit	1:2000	H-V006	Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA
VIP	Rabbit	1:2000	ab22736	Abcam, Cambridge, United Kingdom
AF 488	Donkey anti-mouse IgG (H + L)	1:1000	A21202	ThermoFisher Scientific, Waltham, MA, USA
AF 546	Donkey anti-rabbit IgG (H + L)	1:1000	A10040	ThermoFisher Scientific, Waltham, MA, USA
AF 546	Goat anti- guinea pig IgG (H + L)	1:1000	A11074	ThermoFisher Scientific, Waltham, MA, USA
AF 546	Goat anti-rat IgG (H + L)	1:1000	A11081	ThermoFisher Scientific, Waltham, MA, USA

PGP 9.5—(panneuronal marker) protein gene product 9.5, CART—cocaine and amphetamine regulated transcript, Gal—galanin, nNOS—neuronal nitric oxide synthase, SP—substance P, VAChT—vesicular acetylcholine transporter, VIP—vasoactive intestinal peptide, AF—Alexa Fluor.

Ultrastructural study

The duodenum samples were immersion-fixed in a mixture of 1% paraformaldehyde and 2.5% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4) for 2 h at 4 °C. Next, they were washed in the buffer and post-fixed in a solution containing 2% aqueous osmium tetroxide and 1.5% potassium ferrocyanide in 0.15 M cacodylate buffer with 2 mM calcium chloride for 1 h, washed in water and placed in freshly prepared, filtered 1% thiocarbohydrazide solution for 20 min. The samples were then rinsed in water, incubated in 2% osmium tetroxide for 30 min, rinsed again in water and placed in 1% aqueous uranyl acetate for overnight incubation at 4°. The following day, the samples were stained according to Walton's lead aspartate method for 30 min, washed in water, dehydrated and embedded in Epon 812. The ultrathin sections were cut using PT3D PowerTome ultramicrotome with ASH2 (Boeckeler Instruments, Inc., Tucson, AR, USA) and collected on silicon wafers. The sections were imaged in hierarchical mode using a Sense BSD backscatter electrons detector in SEM Gemini 450 controlled by Atlas 5 software (Carl Zeiss, Oberkochen Germany).

Statistical analysis

The assumption of linearity and normality was checked before statistical analysis. To study linearity, two-dimensional scatter plots of the analyzed variables were generated. The assumption of normality was validated using histograms and residual normality plots. The following values were calculated for the obtained results: mean (*M*), standard deviation (*SD*) and standard error of the mean *(SEM*). A one-way analysis of variance (ANOVA) was used to show statistically significant differences between the number of neurons immunoreactive to CART, GAL, nNOS, SP, VAChT, VIP, length of villi, deep of crypts, the thickness of mucosa, submucosa and muscularis externa (dependent variables) in individual research groups (qualitative variable). The homogeneity of variance was determined using Levene's test before ANOVA. Post-hoc analysis (Scheffe test) was used to assess statistically significant differences between the individual study groups. Differences were considered statistically significant at $p < 0.05$. Statistical analysis of the obtained results was carried out using the Statistica 13.3 program (TIBCO Software Inc., Palo Alto, USA).

Abbreviations

AF	Alexa Fluor
C	Control group
CART	Cocaine and amphetamine regulated transcript
df	Degrees of freedom
eNOS	Endothelial nitric oxide synthase
ENS	Enteric nervous system
f	Fisher F ratio
GAL	Galanin
GALT	Lymphatic tissue associated with the gastrointestinal tract
HD	High dose group
IBD	Inflammatory bowel disease
IL	Interleukins
iNOS	Inducible nitric oxide synthase
ISP	Inner submucous plexus
LD	Low dose group
MP	Myenteric plexus
MS	Mean squares
nNOS	Neuronal nitric oxide synthase
OSP	Outer submucous plexus
PBS	Phosphate-buffered saline
PET	Polyethylene terephthalate
PET-MP	Polyethylene terephthalate microplastics
PGP 9.5	Protein gene product 9.5
SP	Substance P
TNF	Tumor necrosis factor
VAChT	Vesicular acetylcholine transporter
VIP	Vasoactive intestinal peptide

Author contributions
IG: Conceptualization, methodology, validation, formal analysis, investigation, resources, writing—original draft, project administration, software, visualization, funding acquisition, writing—review and editing, NS: Methodology, formal analysis, visualization JC: Writing—review and editing, supervision, funding acquisition.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Procedures were carried out respecting the relevant Polish and EU legislation concerning Animal Protection and Welfare (Leg. Decree 26/2014 implementing the EU directive 2010/63/EU). Written informed consent to use the animals in our procedures was obtained from the owner. The procedures were approved and permission for specimens collection granted by the Local Ethics Committee of the University of Warmia and Mazury in Olsztyn (decision no. 10/2020 of 26 February 2020), affiliated to the National Ethics Commission for animal experimentation (Polish Ministry of Science and Higher Education).

Consent for publication
Not applicable.

Competing interests
The authors declare they have no competing interests.

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Oral Exposure to Microplastics Affects the Neurochemical Plasticity of Reactive Neurons in the Porcine Jejunum

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Abstract: Plastics are present in almost every aspect of our lives. Polyethylene terephthalate (PET) is commonly used in the food industry. Microparticles can contaminate food and drinks, posing a threat to consumers. The presented study aims to determine the effect of microparticles of PET on the population of neurons positive for selected neurotransmitters in the enteric nervous system of the jejunum and histological structure. An amount of 15 pigs were divided into three groups (control, receiving 0.1 g, and 1 g/day/animal orally). After 28 days, fragments of the jejunum were collected for immunofluorescence and histological examination. The obtained results show that histological changes (injury of the apical parts of the villi, accumulations of cellular debris and mucus, eosinophil infiltration, and hyperaemia) were more pronounced in pigs receiving a higher dose of microparticles. The effect on neuronal nitric oxide synthase-, and substance P-positive neurons, depends on the examined plexus and the dose of microparticles. An increase in the percentage of galanin-positive neurons and a decrease in cocaine and amphetamine-regulated transcript-, vesicular acetylcholine transporter-, and vasoactive intestinal peptide-positive neurons do not show such relationships. The present study shows that microparticles can potentially have neurotoxic and pro-inflammatory effects, but there is a need for further research to determine the mechanism of this process and possible further effects.

Keywords: enteric nervous system; microparticles; plastic; polyethylene terephthalate; small intestine; gastrointestinal tract; food chain

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1. Introduction

The jejunum is the section of the small intestine that has the highest length in relation to other sections of the digestive tract. This is where the essential digestion and absorption of nutrients takes place. Its proper function is mediated inter alia by the enteric nervous system (ENS), which regulates food intake, enzyme and electrolyte secretion, blood flow, and immune response [1–3]. It is made up of a dense network of nerve connections that are concentrated in two main plexuses. The myenteric plexus (Auerbach's plexus) is located between the circular and the longitudinal layers of smooth muscle along the entire length of the gastrointestinal tract, i.e., from the oesophagus to the anus [4,5]. The submucous plexus (Meissner's plexus) is located in the mucous membrane, mainly in the small and large intestine [1,3–6]. The number of plexuses is determined by the animal species and the section of the digestive tract [3,5].

Both plexuses exhibit the capacity to regulate the secretory and motor functions of the gastrointestinal tract [3,7]. The myenteric plexus (MP) is mainly responsible for the passage of food content through the digestive tract [7]. The inhibitory neurotransmitters, responsible for the relaxation of the intestinal muscle layer, mainly include vasoactive intestinal peptide (VIP), nitric oxide (NO), pituitary adenylate cyclase-activating peptide

(PACAP), and purines, whereas the stimulatory action is mainly exhibited by tachykinins (including, e.g., neurokinin A (NKA), substance P (SP)), acetylcholine (ACh), or galanin (GAL) [4,8]. In turn, the submucous plexus neurons mainly regulate epithelial secretory functions and blood flow [2,7]. In pigs, the situation is similar to humans; the submucous plexus can be divided into two parts, namely the outer submucous plexus (OSP) located near the circular muscle, and the inner submucous plexus (ISP) located near the lamina propria of the mucous membrane [3,6]. VIP and ACh are the main neurotransmitters of the secretomotor neurons of this plexus [9]. Similar to MP, ACh mainly exhibits excitatory action, whereas NO exhibits inhibitory action on the smooth muscle as well as the epithelial and enteroendocrine cells [6].

The digestive tract is one of the barriers against the external environment. In addition to food, bacteria, viruses, and contaminants such as heavy metals or plastic microparticles can enter the body. The latter have been recently posing a potential threat to humans, animals, or the natural environment [10–13]. Available data indicate that only approximately 9% of plastics are recycled, with the remainder penetrating into the natural environment [12], where, under the influence of chemical and physical factors, nano- and microparticles are formed. Pinlova et al. estimate that up to 480 million particles can be formed from a 0.5 litre PET (polyethylene terephthalate) bottle [14], which is likely to be a significant risk factor later on. This is continuing to emerge, as microplastics are ubiquitous [12,15–18]. Determining the exact daily dose to which a person standard consumer is exposed is difficult to evaluate. It depends on several factors, such as dietary preferences and age. It should also be taken into account that not only the alimentary route, but also, for example, the inhalation of microplastics from the air can represent a gateway of entry into the living organism. Senathirajah et al. estimated the exposure to microplastics to be between 0.1 and 5 g of microplastics/week/person [19], which translates into an average of between 0.2 and 10.2 mg/kg of body weight/day [20]. To date, the presence of microplastics in humans has been confirmed in faeces, colon, placenta, breast milk, lungs, bronchoalveolar fluid, sputum, blood, spleen, liver, and kidneys [13,17,21]. The oral and inhalation routes, and, to a lesser extent, the dermal route, are considered to be the main routes of exposure in humans [13,18,21].

As plastics are widely used in the agri-food sector, the food chain is a critical point of consumers' exposure to its nano- and microparticles. Feed may be a source of pigs' exposure to microplastics [22], as evidenced by their subsequent occurrence in their faeces [23]. The processing of food products [24], or storing them in containers made of plastic, can contribute to the entry of microparticles into the body [12,18,25]. The microplastics chosen for the experiment were polyethylene terephthalate (MP-PET) that are used in the food industry for packing food and beverages. In addition, the studies presented in the available literature are dominated by other types of plastics [17,26–29], which is why the investigation of the impact of PET fills an existing knowledge gap.

The aim of the current study was to determine the percentage of neurons positive in relation to selected neuronally active substances (cocaine- and amphetamine-regulated transcript (CART), galanin (GAL), neuronal nitric oxide synthase (nNOS), substance P (SP), vesicular acetylcholine transporter (VAChT), and vasoactive intestinal peptide (VIP)) and histological changes in the jejunum of the domestic pig after a 28-day exposure to MP-PET at a dose of 0.1 g/day/animal and 1 g/day/animal in order to better understand the effects of microplastics on the ENS. Previous results regarding a different part of the gastrointestinal tract study from the same animals were published by Gałęcka et al. [30].

2. Materials and Methods

2.1. Animals

The study used 15 sexually immature Pietrain × Duroc gilts from a farm in Lubawa (Poland), which weighed approximately 20 kg (Figure 1) and were eight weeks old at the start of the experiment. The animals were kept in the premises of the Faculty of Veterinary Medicine of the University of Warmia and Mazury in Olsztyn under the following condi-

tions: a temperature of 20–22 °C, relative humidity of 55–60%, and a 12 h light–dark cycle. The animals were fed twice a day with commercial feed and had access to water ad libitum. Before introducing the animals, plastic objects were removed from the surroundings. Feeders were made from stainless steel. Before the experiment started, a 7-day acclimatisation in the new environment was carried out. The gilts were randomly divided into three study groups of five individuals already at the acclimatisation stage. Animals were randomised using a computer based random order generator. The handling of the animals and all the procedures were carried out in accordance with Polish laws (Journal of Laws 2015, No 266), which lay down the conditions and methods for carrying out experiments on animals, 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 experiment protocols were submitted to and approved by the Local Scientific Research Ethics Committee of the University of Warmia and Mazury in Olsztyn (Decision No. 10/2020 of 26 February 2020).

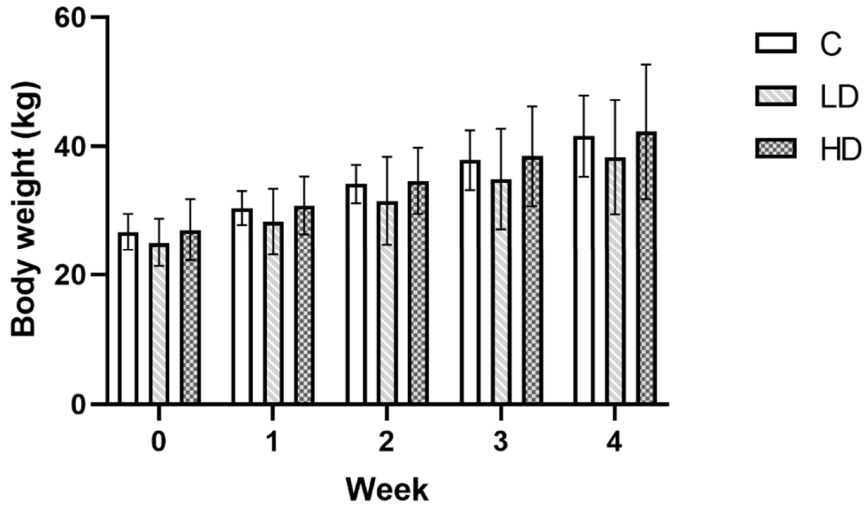


Figure 1. Bodyweight of gilts in weekly measurements. There were no statistically significant differences between groups in weeks 0, 1, 2, 3, and 4 ($p > 0.05$).

2.2. Experimental Procedures

The gilts were divided into three groups: a control group (C; $n = 5$), a group which received a low dose (LD; $n = 5$) of 0.1 g of MP-PET/day/animal, and a group which received a high dose (HD; $n = 5$) of 1 g of MP-PET/day/animal for 28 days. Animal handling always started with group C, then LD, and then HD. The animal keeper was not aware of the treatment group allocation. The microparticles were administered once daily, orally by capsule applicator, one hour before the morning feeding, in gelatine capsules. Animals in group C were administered empty capsules, whereas those in groups LD and HD were administered capsules with microparticles of polyethylene terephthalate (Cat. No ES306031/1, Goodfellow Cambridge Ltd., Huntingdon, UK). The MP-PET particles were heterogeneously shaped. Particle size was determined using laser diffraction analysis with a Mastersizer 2000 analyzer (Malvern Instruments Ltd., Malvern, UK). The obtained results indicate that the analysed sample contains particles in the range of 7.6 µm–416.9 µm, with the dominant share of particles with a diameter of 158.5 µm. Analysis of the results shows that in this sample 10% of particles have diameters no greater than 51.6 µm, 50% have diameters no greater than 124.6 µm, and 90% have diameters smaller than 237.0 µm. The mean diameter of particles D [3,4] in the sample is 135.6 µm Figure 2A. Microscopic analysis of particles was performed using a Phenom ProX G6 scanning microscope (ThermoFisher Scientific, Waltham, MA, USA) (Figure 2B,C). The applied dose of 0.1 g/day/animal was adjusted, based on a study by Deng et al. [31], to the weight of the gilts, whereas the dose of 1 g/day/animal was a 10-fold higher dose (calculated on a logarithmic scale—the logarithm of the decimal (for $\log(0.1) = -1$; for $\log(1) = 0$)), and was of an overloading nature.

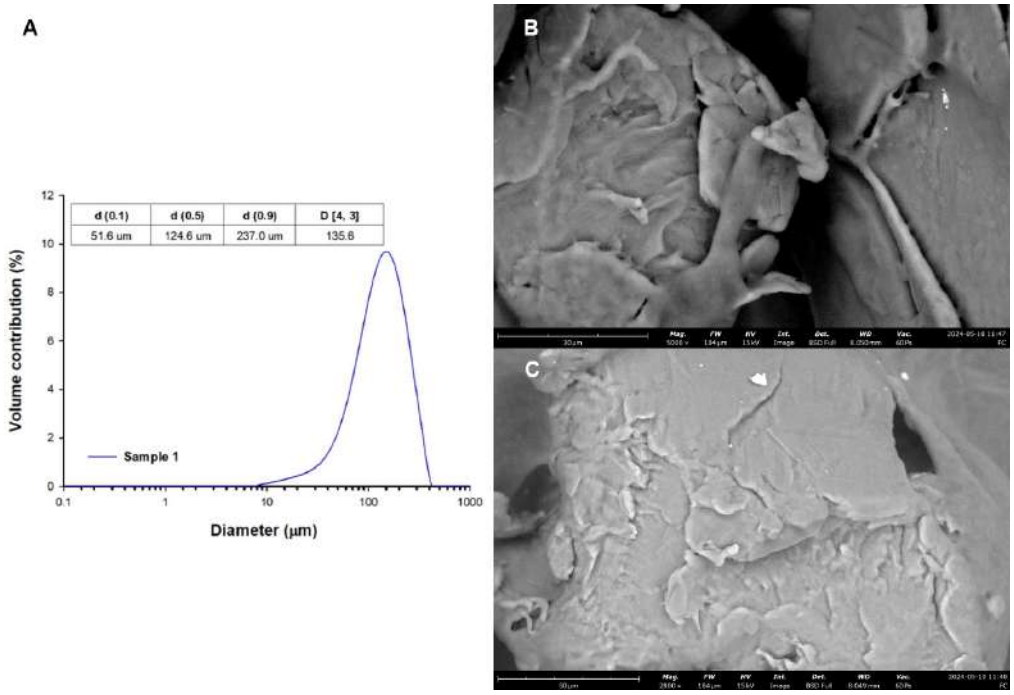


Figure 2. (**A**) Particle size distribution and (**B**,**C**) SEM images.

2.3. Tissue Sampling

After 28 days, the animals were euthanised. The euthanasia protocol was based on the use of atropine (0.05 mg/kg i.m., Polfa, Warszawa, Poland), followed by xylazine (3 mg/kg i.m., Vet-Agro, Lublin, Poland) and ketamine (6 mg/kg i.m., Vetoquinol Biowet, Gorzów Wielkopolski, Poland). After approximately 20 min, when the gilts were unconscious, an overdose of sodium pentobarbital (0.6 mL/kg i.v., Biowet, Puławy, Poland) was applied. Once the cessation of vital functions was confirmed (no pulse, no respiration, no corneal reflex, cardiac rhythm disappeared, and respiration ceased), sampling for further tests began immediately. An approximately 8-cm-long section of the jejunum was taken, a section located 100 cm from the pylorus of the stomach. The tissues were immediately fixed in order to carry out double immunofluorescence staining and histological examination.

2.4. Double Immunofluorescence Staining

In order to fix the samples, the collected sections of the jejunum were placed in a 4% paraformaldehyde solution (pH 7.4) for one hour and then rinsed for three consecutive days with 0.1 M phosphate buffer (pH 7.4), with the buffer being changed every 24 h. After three days, the tissues were transferred to an 18% sucrose solution (pH 7.4) for two weeks at a temperature of 4 °C. Once fixed, sections of the jejunum were embedded in OCT Tissue-Tek (Sakura Finerek USA, Inc., Torrance, CA, USA) and, using a cryostat (CM 1860, Leica, Nussloch, Germany), cut at a temperature of −22 °C into sections with a thickness of 14 µm, and attached to chrome-alum-coated slides. The staining procedure lasted for two days, (1) the slides being first dried at room temperature for 45 min, (2) followed by rinsing with 0.1 M phosphate-buffered saline (PBS, pH 7.4, 3 × 15 min). (3) The next step involved incubation with a blocking mixture (10% horse serum and 0.1% bovine serum albumin in 0.1 M PBS, 1% Triton X-100, 0.05% Thimerosal, and 0.01% sodium azide) for 1 h at room temperature in a humid chamber. (4) The blocking mixture was washed out using PBS solution (3 × 15 min). (5) A mixture of primary antibodies consisting of protein gene product 9.5 (PGP 9.5, a pan-neuronal marker, used to visualise neurons in the jejunum) and against one of the test substances (CART/GAL/nNOS/SP/VAChT/VIP) was applied onto the intestinal sections overnight. (6) On the second day of staining, the sections were washed out with PBS solution (3 × 15 min). (7) Secondary antibodies Alexa Fluor 488 (to visualise PGP 9.5 posi-

tive neurons) and 546 (to visualise CART/GAL/nNOS/SP/VAChT/VIP positive neurons) were applied onto the tissues for 1 h in a humid chamber. The list of antibodies used in immunofluorescence method is presented in Table 1. (8) The final staining step involved rinsing with PBS solution (3 × 15 min), (9) coating the tissues with carbonate-buffered glycerol (pH 8.6), and (10) covering them with a cover slip. Routine follow-up antibody tests were performed to exclude non-specific staining. These tests involved the pre-absorption of antibodies with the relevant antigen, the omission test, and the replacement of primary antibodies with non-immune sera. The stained intestinal fragments were analysed using a Zeiss Axio Imager.M2 fluorescence microscope (Zeiss, Oberkochen, Germany) equipped with the appropriate set of filters. At least 500 PGP 9.5-positive neurons (green) with a clearly visible nucleus in each of the three plexuses (MP, OSP, and ISP) were counted as 100% of the population. By changing the filter, the neurons positive to the test substances (red) were then visualised, counted, and determined as a percentage of PGP 9.5-positive neurons. In order to avoid counting the same neurons, the distance between the fragments was at least 200 μm.

Table 1. List of antibodies used in immunofluorescence method.

Antigen	Host Species	Final Dilution	Code	Supplier
Primary Antibodies				
Protein gene product 9.5	Mouse	1:1000	480012	ThermoFisher Scientific, Waltham, MA, USA
Neuronal nitric oxide synthase	Rabbit	1:3000	PA1-033	
Cocaine- and amphetamine-regulated transcript	Rabbit	1:16,000	H-003-61	Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA
Vesicular acetylcholine transporter	Rabbit	1:2000	H-V006	
Galanin	Guinea pig	1:2000	T-5036	Peninsula, San Carlos, CA, USA
Substance P	Rat	1:150	8450-0505	BioRad, Hercules, CA, USA
Vasoactive intestinal peptide	Rabbit	1:2000	ab22736	Abcam, Cambridge, UK
Secondary Antibodies				
Alexa Fluor 488	Donkey anti-mouse IgG (H + L)	1:1000	A21202	ThermoFisher Scientific, Waltham, MA, USA
Alexa Fluor 546	Donkey anti-rabbit IgG (H + L)		A10040	
Alexa Fluor 546	Goat anti-guinea pig IgG (H + L)		A11074	
Alexa Fluor 546	Goat anti-rat IgG (H + L)		A11081	

2.5. Histological Examinations

The samples of jejunum were fixed in 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4) for 48 h, dehydrated in ethanol in a tissue infiltration machine (Leica TP1020, Leica, Nussloch, Germany), and embedded in paraffin (EG1150, Leica, Nussloch, Germany). The 4 μm-thick sections were prepared with microtome (HM 340E, Microm, Spain) and stained with the haematoxylin and eosin method (HE) using a multistainer (ST5020 + CV5030, Leica, Nussloch Germany). The slides were digitalised in a Pannoramic 250 Flash scanner (3DHistech, Budapest, Hungary). Morphometric analyses were performed using SlideViewer 2.6 software (3DHistech, Budapest, Hungary) and were included the length of villi, the crypt depth, the thickness of mucosa, the thickness of submucosa, and the thickness of muscularis externa. The measurements were performed on three sections per animal, separated each other by at least 1 cm, in 10 replicates per slide, and the mean values were subjected to a statistical analysis.

2.6. Statistical Analysis

Prior to statistical analysis, the assumption of linearity and normality was checked. For linearity testing, two-dimensional scatter plots of the analysed variables were generated. The assumption of normality was confirmed using histograms and residual normality plots. The following values were calculated from the results obtained as follows: the mean (M), the standard deviation (SD), and the standard error of the mean (SEM). One-way analysis of variance (ANOVA) was applied to demonstrate statistically significant differences between the number of neurons immunoreactive to CART, GAL, nNOS, SP, VAChT, and VIP (dependent variable) in each study group (qualitative variable).

Analysis of variance was used for statistically significant indices between the morphometric indices (dependent variables) in individual study groups (qualitative variable). The homogeneity of variance was determined using Levene's test before the ANOVA. A post hoc analysis (Scheffe's test) was used to assess statistically significant differences between individual study groups. The differences were considered statistically significant at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). A statistical analysis of the results was conducted using Statistica 13.3 software (TIBCO Software Inc., Palo Alto, USA), whereas the visualisation was carried out using GraphPad Prism 9.0.0 software (GraphPad Software, Boston, MA, USA).

3. Results

3.1. Myenteric Plexus

Under physiological conditions, the most abundant population of all the tested substances was that of nNOS-positive neurons, standing at 25.75 ± 2.78% (C), which decreased to 14.77 ± 3.02% under the influence of HD MP-PET. Cholinergic neurons represented by VAChT 25.45 ± 1.39% (C), the percentage of which also decreased to 20.06 ± 1.13% under the influence of HD, were comparably abundant. LD MP-PET did not contribute to the emergence of statistically significant differences. LD and HD MP-PET also reduced the population of CART-positive neurons from 9.38 ± 1.64% (C) to 4.32 ± 0.37% (LD) and 4.95 ± 0.77% (HD), the population of VIP-positive neurons from 15.46 ± 0.98% (C) to 7.50 ± 0.64% (LD) and 5.23 ± 1.04% (HD), and that of SP-positive neurons from 4.97 ± 1.66% (C) to 1.83 ± 0.50% (LD) and 2.13 ± 0.62% (HD). An increase in the neuronal population only occurred for GAL, where an increase from 3.40 ± 1.08% (C) to 6.63 ± 0.83% (LD) and 11.28 ± 1.86% (HD) could be observed. It should be noted that only the MP showed a reduction in the percentage of SP-positive neurons, in contrast to the two submucous plexuses, where an increase occurred. The data are provided in Figures 3A and 4.

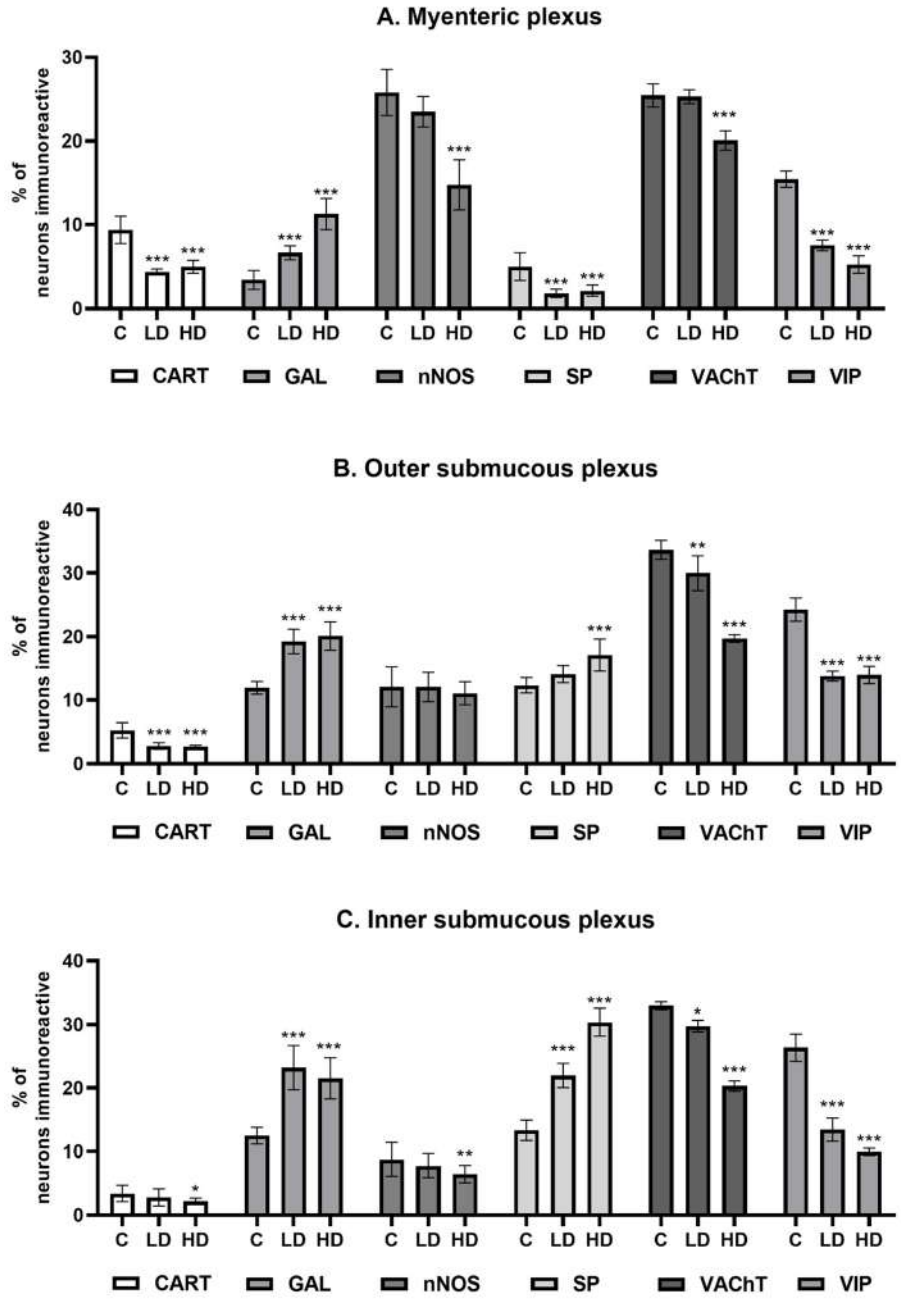


Figure 3. Percentage of neurons immunoreactive (**A**) in the myenteric plexus, (**B**) outer submucous plexus, and (**C**) inner submucous plexus. Percentage (Mean ± SEM) of neurons immunoreactive for cocaine- and amphetamine-regulated transcript peptide (CART), galanin (GAL), neuronal nitric oxide synthase (nNOS), substance P (SP), vesicular acetylcholine transporter (VAChT), and vasoactive intestinal polypeptide (VIP) of all neuronal cells immunoreactive to PGP 9.5 in the control group (**C**), low-dose group (LD), and high-dose group (HD) of MP-PET. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate statistically significant differences in the expression of the tested substances in relation to the control group.

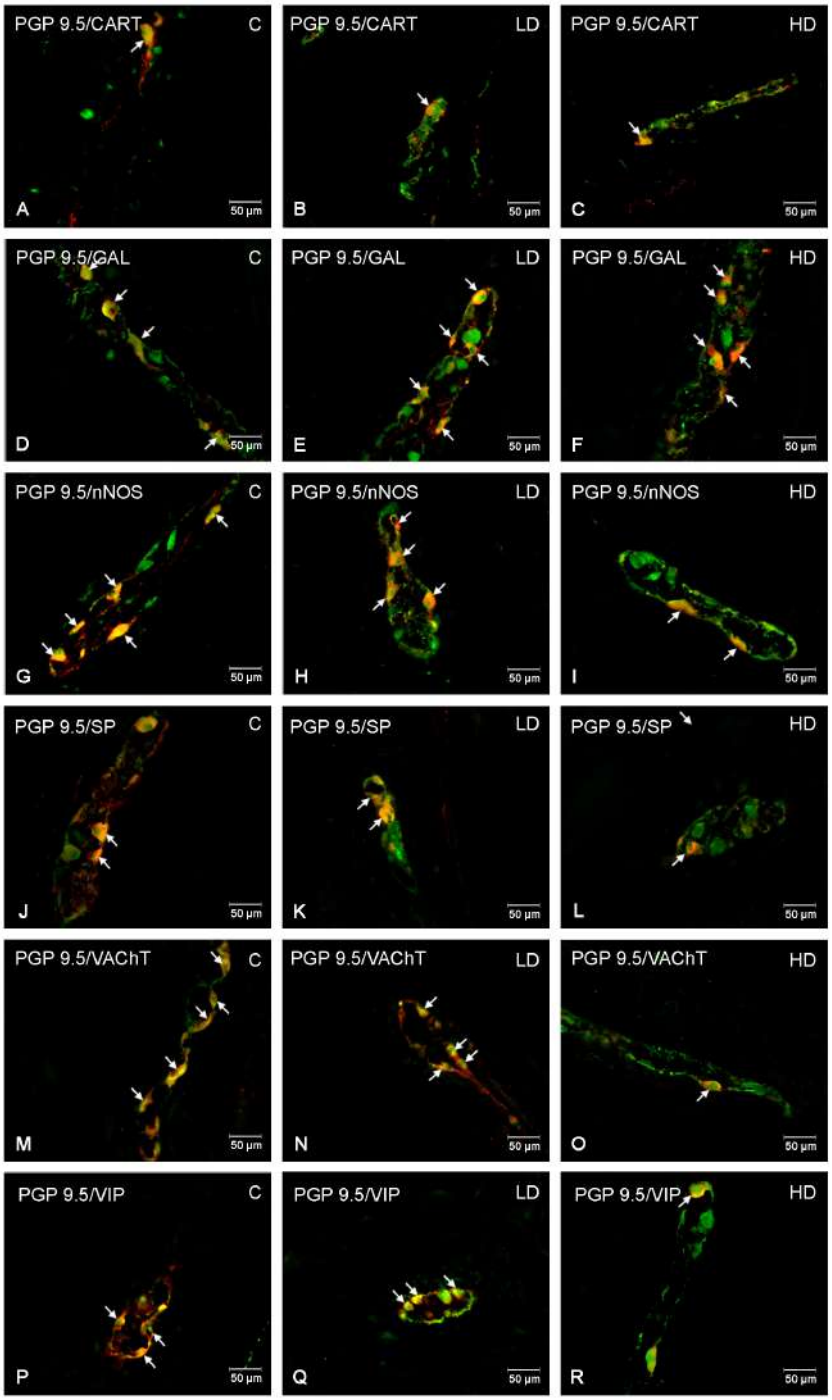


Figure 4. The myenteric plexus of porcine jejunum. Fluorescence microscope image showing the distribution of neurons immunoreactive to PGP 9.5—used as a pan-neuronal marker and CART, GAL, nNOS, SP, VAChT, VIP in the control group—C (**A**,**D**,**G**,**J**,**M**,**P**), after administration of low (**B**,**E**,**H**,**K**,**N**,**Q**) and high doses (**C**,**F**,**I**,**L**,**O**,**R**) of MP-PET. All photographs were made by overlapping green and red fluorescent channels (green for PGP 9.5 and red for CART, GAL, nNOS, SP, VAChT, and VIP, respectively). Neurons immuno-positive to a particular substance studied are indicated with arrows.

3.2. Outer Submucous Plexus

Cholinergic neurons and VIP-positive neurons jointly accounted for almost 58% of all neurons in the OSP under physiological conditions. In both cases, following exposure to MP-PET, a decrease was observed in the percentage of VAChT-positive neurons from 33.63 ± 1.53% (C) to 29.99 ± 2.69% (LD) and 19.71 ± 0.60% (HD), and for VIP-positive

neurons from 24.28 ± 1.84% (C) to 13.77 ± 0.78% (LD) and 13.95 ± 1.38% (HD). The population of CART-positive neurons also decreased from 5.27 ± 1.20% (C) to 2.80 ± 0.53% (LD) and 2.67 ± 0.26% (HD). The percentage of GAL-positive neurons increased from 11.90 ± 1.00% (C) to 19.24 ± 1.93% (LD) and 20.10 ± 2.25% (HD). The population of SP-positive neurons increased from 12.32 ± 1.22% (C) to 17.10 ± 2.52% (HD). No statistically significant differences induced by LD MP-PET were observed. Interestingly, neither LD nor HD MP-PET contributed to a change in the population size of nNOS-positive neurons. The data are provided in Figures 3B and 5.

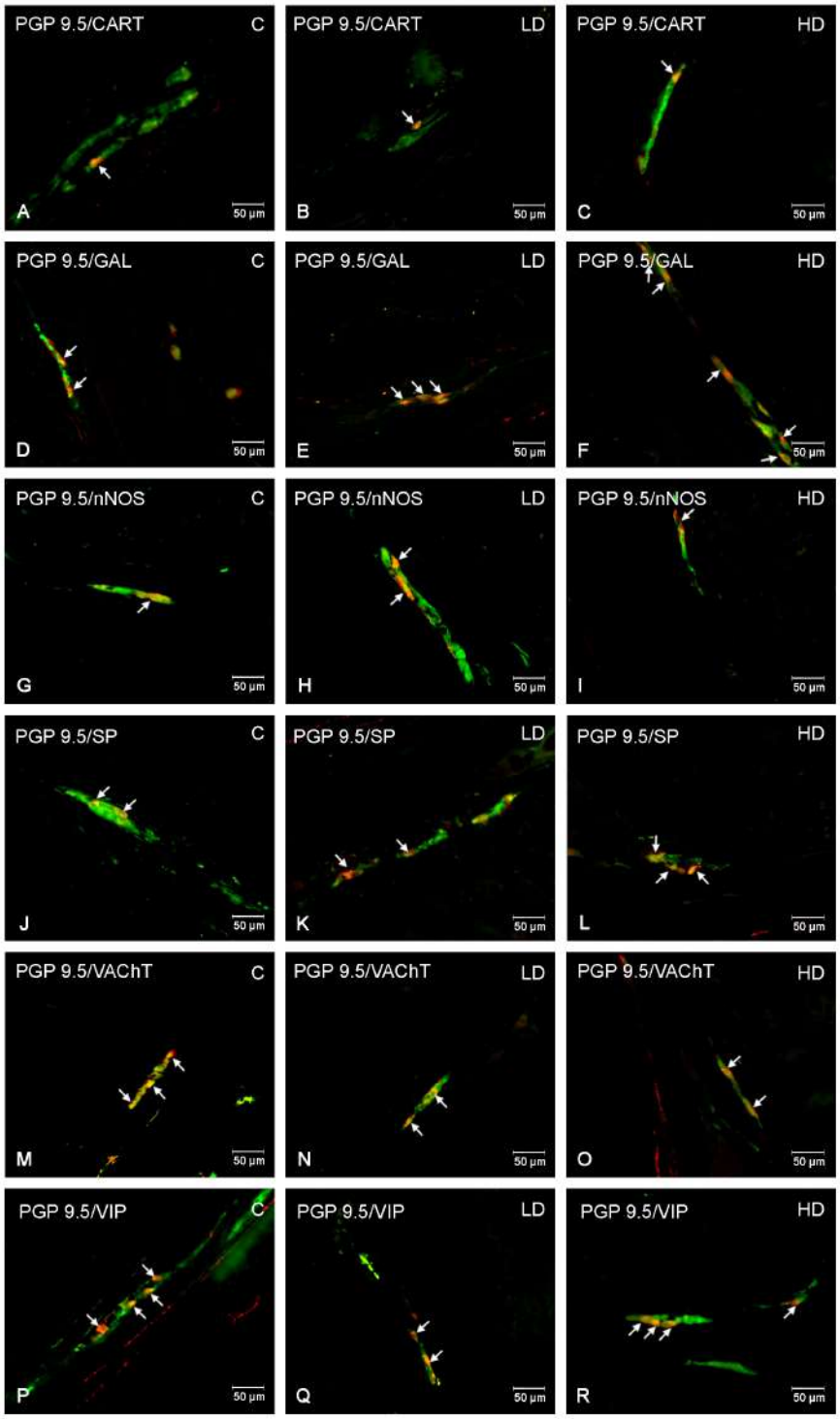


Figure 5. The outer submucous plexus of porcine jejunum. Fluorescence microscope image showing the distribution of neurons immunoreactive to PGP 9.5 —used as a pan-neuronal marker and CART,

GAL, nNOS, SP, VAChT, and VIP in the control group—C (**A**,**D**,**G**,**J**,**M**,**P**), after administration of low (**B**,**E**,**H**,**K**,**N**,**Q**) and high doses (**C**,**F**,**I**,**L**,**O**,**R**) of MP-PET. All photographs were made by overlapping green and red fluorescent channels (green for PGP 9.5 and red for CART, GAL, nNOS, SP, VAChT, and VIP, respectively). Neurons immuno-positive to a particular substance studied are indicated with arrows.

3.3. Inner Submucous Plexus

Similar to the previously described plexus, VIP- and VAChT-positive neurons represented the most abundant population under physiological conditions. The population of VAChT-positive neurons decreased from 32.91 ± 0.63% (C) to 29.76 ± 0.91% (LD) and 20.30 ± 0.82% (HD), whereas that of VIP-positive neurons decreased from 26.37 ± 2.14% (C) to 13.41 ± 1.84% (LD) and 9.92 ± 0.60% (HD). A more than two-fold increase in SP-positive neurons under the influence of HD, from 13.30 ± 1.60% (C) to 30.35 ± 2.15% (HD), was noted. The low dose contributed to an increase in the population of these neurons, but to a lesser extent, being 21.96 ± 1.92% (LD). An increase in the percentage of GAL-positive neurons was also noted from 12.49 ± 1.30% (C) to 23.22 ± 3.49% (LD) and 21.52 ± 3.25% (HD). LD MP-PET did not contribute to changes in the population of CART-positive and nNOS-positive neurons, in contrast to HD. The percentage of CART-positive neurons decreased from 3.42 ± 1.28% (C) to 2.19 ± 0.51% (HD), whereas the population of nitrergic neurons (nNOS) decreased from 8.76 ± 2.66% (C) to 6.48 ± 1.38% (HD). The data are provided in Figures 3C and 6.

3.4. Histological Examinations

The jejunum of control animals was characterised by the presence of irregularly shaped villi of moderate length, which were covered by the columnar epithelium comprising mostly adsorptive enterocytes and much fewer numerous goblet cells (Figure 7A). The intestinal glands had the form of abundant tortuous tubes (Figure 7A). The lamina propria contained lymphocytes, plasma cells, and eosinophils. The muscularis mucosae was well developed. Villi with damaged apical parts were infrequently observed in control animals.

The desquamation of enterocytes leading to injury of the apical parts of the villi was frequently observed in the jejunum samples from the animals treated with LD (Figure 7B) and HD of MP-PET (Figure 7C). The loss of enterocytes or the presence of an abnormal epithelium was also noted on the lateral surfaces of the villi (Figure 7C). The large accumulations of cellular debris and mucus occurred in folds of the mucosa (Figure 8A). Strong eosinophil infiltration (Figure 8B) and hyperaemia (Figure 8C) were observed in the lamina propria in both experimental groups. The pathological changes were more prominent in the pigs receiving higher doses of PET.

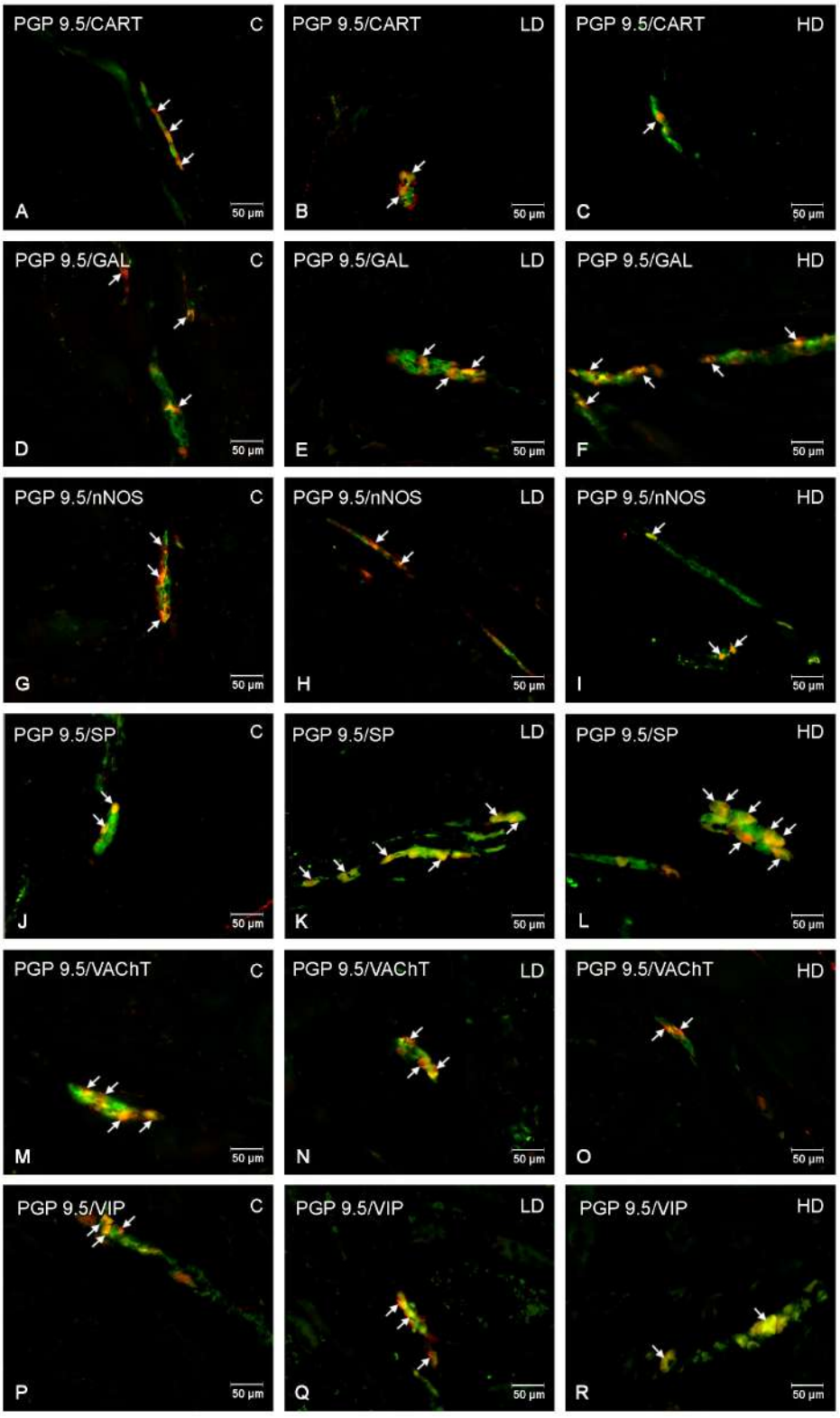


Figure 6. The inner submucous plexus of porcine jejunum. Fluorescence microscope image showing the distribution of neurons immunoreactive to PGP 9.5 —used as a pan-neuronal marker and CART, GAL, nNOS, SP, VAChT, and VIP in the control group—C (**A**,**D**,**G**,**J**,**M**,**P**), after administration of low (**B**,**E**,**H**,**K**,**N**,**Q**) and high doses (**C**,**F**,**I**,**L**,**O**,**R**) of MP-PET. All photographs were made by overlapping green and red fluorescent channels (green for PGP 9.5 and red for CART, GAL, nNOS, SP, VAChT, and VIP, respectively). Neurons immuno-positive to a particular substance studied are indicated with arrows.

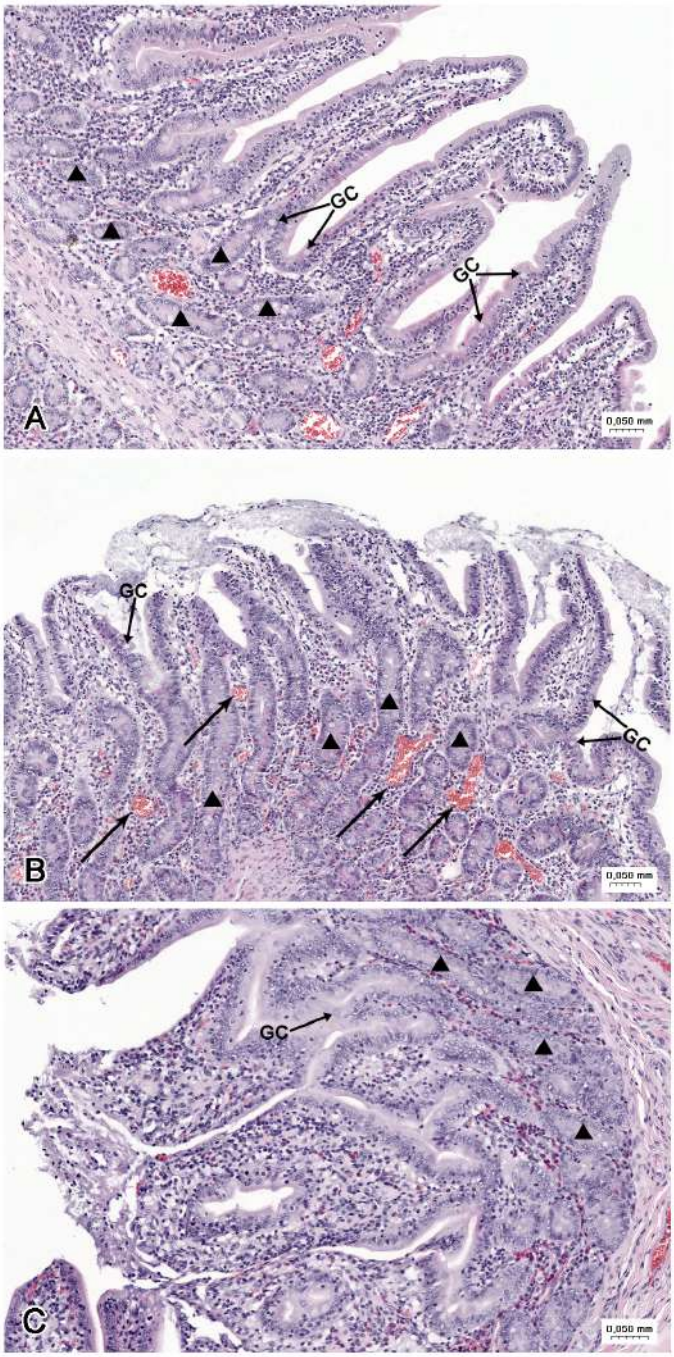


Figure 7. Histological structure of the jejunum (**A**) in the C group, (**B**) in the LD group, and (**C**) the HD group. (**A**) Tunica mucosa with the villi covered by continuous columnar epithelium. (**B**) Desquamation of enterocytes leading to injury of the apical parts of villi. Note the numerous blood-containing vessels (arrows). (**C**) Loss of enterocytes on the apical and lateral surfaces of the villi. GC—goblet cells, triangle—intestinal glands.

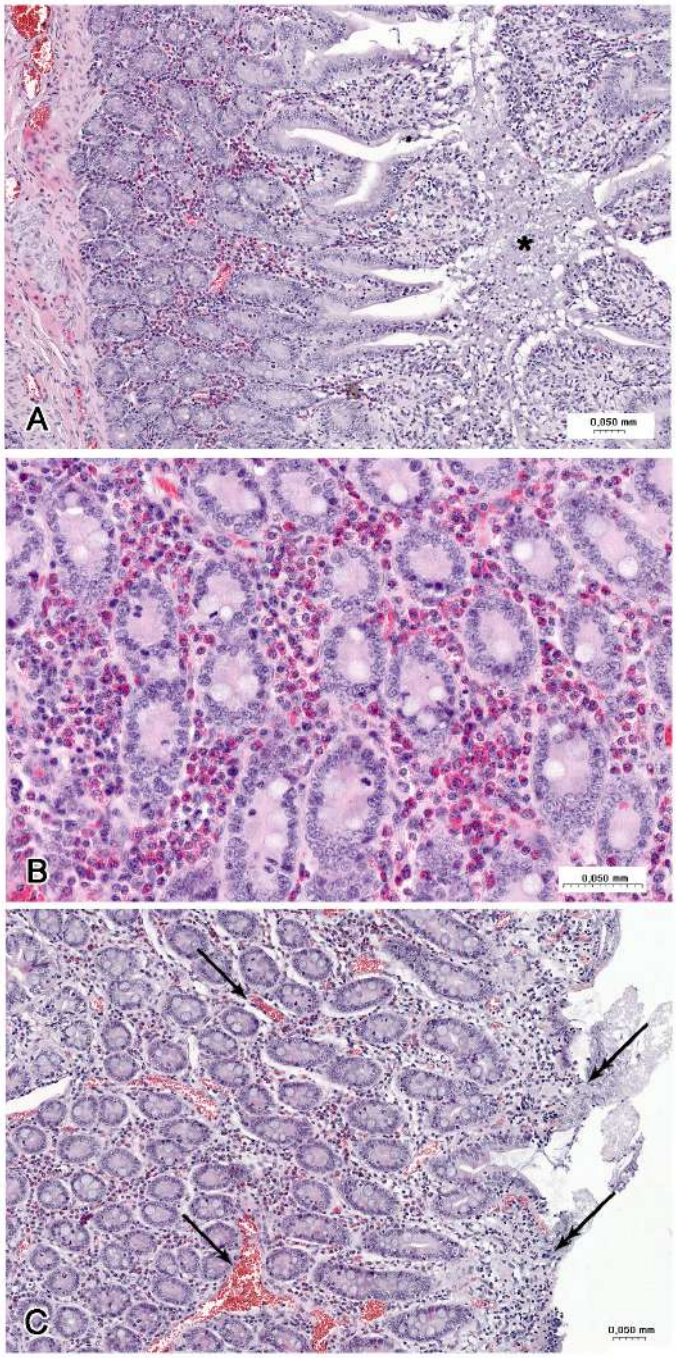


Figure 8. Histological structure of the jejunum (**B**) in a LD group and (**A**,**C**) HD group. (**A**) Accumulation of cellular debris and mucous (asterisk) in fold of mucosa. Note the hyperaemia and strong infiltration by eosinophils. (**B**) Numerous eosinophils in the lamina propria between the intestinal glands. (**C**) Numerous blood-containing vessels (arrows) in the lamina propria and damage of the villi (double arrows).

The morphometric analysis showed that the length of villi and the thickness of mucosa were significantly lower in the LD and HD groups compared to the C groups. The depth of crypts, as well as the thickness of the submucosa and the muscularis externa, did not differ between the groups (Figure 9).

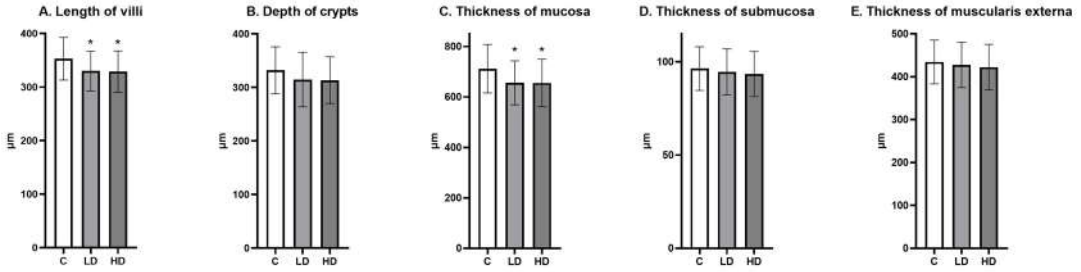


Figure 9. (**A**) The length of villi, (**B**) the depth of crypts, (**C**) the thickness of mucosa, (**D**) the thickness of submucosa, and (**E**) the thickness of the muscularis externa in the jejunum (Mean ± SD). * $p < 0.05$ indicate statistically significant differences in the expression of the tested substances in relation to the control group in the LD group and HD group.

4. Discussion

The study presented in this article confirms that microplastic, as a xenobiotic, affects neuronal populations within the ENS of the jejunum. However, the mechanism of affecting neurons and other cells has not been fully understood. Neurotoxicity can be linked to the ability of particles to accumulate in the mitochondria, leading to mitochondrial dysfunction [32], the induction of oxidative stress [26,33], direct physical damage [28,34], altered neurotransmitter levels [26,30], or disruption of the gut microbiome [33–35], inducing pro-apoptotic protein expression (BAX, caspase 3, caspase 8, caspase 9, DR5, and cytochrome c) and pro-inflammatory cytokines (IL-8, NF-κB and TNF-α) [36]. Up to date, most studies confirm that it is the size of particles that is of greatest importance [26,37]. A size of 10 µm is considered to be the upper limit for microplastic uptake by the cells through phagocytosis, pinocytosis or macropinocytosis [38,39]. Particles larger than 150 µm can be bound to the intestinal mucus layer, whereas smaller particles are capable of penetrating this layer [40], leading to changes in the histological structure [10,34,40,41]. Since the potential effect caused by oral exposure to microplastics is determined by the plastic used, the results obtained may vary, and further research needs to be carried out using other types of microparticles as well as mixtures of different plastics. Another factor influencing the results obtained is the use of primary microplastics, i.e., those free of environmental contaminants. Environmental microplastics can be vectors for toxins, pathogens, and contaminants [28,41,42], and it is these that could potentially pose a greater threat than the microplastics themselves and cause adverse effects in humans and animals. However, more studies comparing the effects of environmental and primary microplastics are needed to prove this hypothesis. The use of microparticles exhibiting a wide variation in shape and size is aimed at approximating the natural conditions, as the actual exposure does not involve standardised particles. A dose of 0.1 g/animal/day is a dose that may indeed reflect daily life, whereas a dose of 1 g/animal/day actually exceeds real-life exposure to microplastics, according to available data [19,20]. However, its application is important in order to learn about the effects of a microplastic overdose.

There are reports available that indicate a high resistance of plastic particles to the action of artificial digestive juices [43], but Krasucka et al. demonstrated that under the influence of digestion, changes in the properties of plastics may occur, including the formation of nanoparticles [42]. Other studies also confirmed that MP-PET could reduce the number of bacterial groups responsible for maintaining the balance of the intestinal microflora and increase the number of bacterial groups capable of inducing a pro-inflammatory state [35]. Polyethylene (PE) microplastics were also observed to exhibit a synergistic action with *Helicobacter pylori*, thus contributing to gastric mucosal damage and inducing gastritis in mice [28]. Interestingly, the previously mentioned study suggests that the colonic microbiome could cause the biodegradation of polyethylene terephthalate [35]. It is known, however, that one of the most frequently described effects of exposure to nano- and microplastics is the induction of intestinal dysbiosis [10,13,40].

Peters et al. studied different types of microplastics (including PET) to find out which chemical substances and metals are released during chemical extraction and when using an in vitro human digestion model [44]. As for PET, the plasticisers bis(2-ethylhexyl) phthalate (DEHP), phthalic acid, di(2-propylpentyl) ester, and tributyl acetylcitrate were identified, as well as the metals lithium, manganese, cobalt, zirconium, tin, antimony, tantalum, and thallium [44]. The authors suggest that exposure to these substances through chemical wash-out when drinking bottled water is low [44], but other studies report that microplastics could be the main source of phthalates in the aqueous environment [45,46]. Further research is needed to determine the extent to which the release of plasticisers from microplastics poses a risk to plastic users.

Based on the available literature, it is known that microplastics can induce oxidative stress [10,13,27,29,39,40,47]. The induction of oxidative stress can be activated by different pathways, for example, by the TLR4/NOX2 signalling axis [48] or the NF-κB/NLRP3/IL-1β/MCLK pathway [49]. In a study on marine organisms, such as mussels, crustaceans, and fish, inhibition of acetylcholinesterase (AChE) activity, which is considered to be an indicator of neuromuscular toxicity [26], was observed. Exposure to PE in mice reduced the ACh levels in the brain tissue by, e.g., inducing oxidative stress [29]. ENS neurons are also sensitive to damage resulting from nitrosative stress, mitochondrial dysfunction, or the presence of inflammation [50]. Studies on mice assessing the effects of microplastics on the large intestinal mucosa demonstrated that they could contribute to the development of mild or moderate colitis, e.g., through intestinal dysbiosis, the occurrence of inflammatory infiltration in the mucosa, increased secretion of pro-inflammatory cytokines, the induction of apoptosis, a reduction in the number of goblet cells, mucin expression, the secretion of mucus, and increased intestinal barrier permeability or tissue accumulation, which corresponds with other available studies [10,13,26,40,41] and the results obtained in this study, especially in the HD group. Available data indicate that microplastics have the potential to exert multiple effects on the ENS as well as on the entire body [17,31,40,51]. The described mechanisms may be the cause of the changes observed in the study presented here.

Neurodegenerative and intestinal diseases pose a growing problem for society. Some of these diseases, e.g., Parkinson's disease or Alzheimer's disease, can have their origin in the ENS [52,53]. With age, under the influence of potentially toxic agents and changes in the intestinal microbiome, changes may occur in the population of ENS neurons and in the chemical coding of neurotransmitters, neuropeptides, and neuroimmunomodulators [52–57].

VIP, NO, and GAL exhibit neuroprotective action [58,59], but it should be noted that an excess of NO can form toxic compounds, namely reactive nitrogen species (RNS), which have already shown a neurodegenerative action [59,60]. Several mechanisms are involved in the neuroprotective properties of NO, including the activation of Akt and CREB (cAMP response element-binding protein) kinase, S-nitrosylation of the NR1 and NR2 subunits of the NMDA (N-methyl-D-aspartate) receptor, decreased Ca^{2+} ion influx, or increased haeme oxygenase expression [60,61].

NO has a similar effect on intestinal motility throughout the gastrointestinal tract, having a relaxing effect and reduces gastric acid secretion in the stomach, but in the duodenum and colon it stimulates the secretory function [59]. ACh stimulates the synthesis of NO, which is subsequently released from the submucosal arteriole endothelial cells, causing a relaxant effect on the vascular smooth muscles and thus regulating the blood flow [7]. Under the influence of bisphenol A [56], acetylsalicylic acid [55], acrylamide [54], and diabetes [62] in the jejunum, as in the present study, there was also a reduction in the percentage of nNOS-positive neurons, with the difference that all the three plexuses were affected. It has been noted that a reduction in the activity of nitric oxide synthase (NOS) occurs during hypoxic-ischaemic encephalopathy as a neuroprotective strategy [63]. Studies conducted on pigs to date also confirm that the highest concentration of nNOS-positive neurons is found in the myenteric plexus [55,56], which is also the case in this study. Interestingly, only in the case of nNOS-positive neurons, a dose of 0.1 g/day/animal (LD) was not observed to induce statistically significant changes in at least one of the plexuses,

whereas a dose of 1 g/day/animal (HD) contributed to the induction of changes only in the MP and ISP, which may indicate, inter alia, an excessive loss of these neurons, e.g., through internal nitrosative damage [50], or a neuroprotective effect through the inhibition of nitric oxide activity. Research has also confirmed that one of the reasons for the reduction in immunoreactivity towards the test substance may be due to the degeneration or necrosis of fibres and neurons, which may be linked to the intensity of inflammation in the intestinal area [64].

The similarities noted in the populations of VIP- and nNOS-positive neurons in the MP may indicate the co-localisation of these neurons, especially as both of them serve a similar function in the regulation of intestinal motility [64,65], and, in addition, both of them may significantly affect neuronal survivability [66]. VIP exhibits neuroprotective action by maintaining a balance between pro- and anti-inflammatory cytokines [67], neuronal survivability [68,69], inhibiting the activity (phagocytosis and chemotaxis) of macrophages [70], stimulating the differentiation of B lymphocytes, and producing IgA [70,71], inhibiting the proliferation and migration of T lymphocytes [70,71], inhibiting the secretion of pro-inflammatory cytokines such as TNF-α, IFNγ, IL-6, IL-12 [69,70], increasing the secretion of IL-10 [69], and inhibiting the inducible nitric oxide synthase (iNOS) [70]. In the gastrointestinal tract, it causes the relaxation of smooth muscles and stimulates the secretion of mucus, intestinal juice, and bile [55,64,71,72]. The percentage of neurons often increases under the influence of inflammatory factors [55,56,67,69] and decreases, e.g., during Parkinson's disease [73]. During inflammatory bowel disease (IBD), an increase or decrease in VIP was observed, depending on the study [53]. The lack of an increase in the percentage of nNOS- and VIP-positive neurons may reflect the fact that neither of these neurotransmitters is involved in the neuroprotection of neurons in the ENS under the influence of MP-PET.

Another substance for which a reduction in the percentage of positive neurons was noted is VAChT, a marker of cholinergic neurons. ACh is transported by VAChT to the synaptic vesicles and is present in the cholinergic nerve endings [7]. In the myenteric plexus, but only in the HD group, a reduced population of VAChT-positive neurons can be noted, which may result in the inhibition of muscle contractile activity, as ACh is considered to be the main substance inducing smooth muscle contractions [74]. Under the influence of both doses of MP-PET, there was a reduction in the percentage of VAChT-positive neurons in the OSP and ISP. This may result in reduced ion secretion [7,72], a lack of inhibition of pro-inflammatory cytokine secretion [68], or a lack of neuroprotective action [75]. During neurodegenerative diseases, both the loss of cholinergic neurons and a reduced percentage of VAChT neurons can be observed [76]. Inflammatory and toxic factors may, however, contribute to an increase [54] or a decrease in VAChT activity [56]. The neurotoxicity of nano- and microplastics has been described in animals such as invertebrates, fish, or rodents [26,29]. As for the studies mentioned, AChE activity was mainly inhibited [26]. A reduction in the percentage of VAChT-positive neurons may be another mechanism contributing to neurotoxicity.

The last substance for which a reduction in all the plexuses was noted is the neuropeptide CART. A study conducted on mice and pigs indicates that CART is primarily found in the stomach, the duodenum, and the jejunum [77]. Current data indicate that CART serves neuroprotective functions [55,77], has an effect on food intake, and reduces both the secretion of gastric juice and motor activity [54,74]. It was noted that in the jejunum, the population of CART-positive neurons was larger in the myenteric plexus than in the submucous plexuses, as reflected in the literature [55,56,78,79]. The population of CART-positive neurons in the jejunum increased under the influence of various agents, e.g., acrylamide [78], bisphenol A [56], and acetylsalicylic acid [55], or decreased in the Huntington's disease [80]. In the duodenum, MP-PET also induced an increase in the population of CART-positive neurons [30]. A similar trend can be observed with the effect of bisphenol A on the small intestine, except that there was a decrease in the duodenum [79], whereas in the jejunum, there was an increase [56] in the percentage of CART-positive neurons.

The latter two substances exhibit increased immunoreactivity under the influence of MP-PET. GAL may exert anti-inflammatory action by increasing the synthesis of IFN-γ and IL-12/23 and decreasing TNF-α and IL-1β [56]. It regulates smooth muscle contraction [79], presumably by inhibiting ACh and SP [72], regulates the secretion and flow of the blood [81], and exhibits neurotrophic properties [67]. In myenteric neuron culture, GAL contributed to a decrease in neuronal survivability [68]. A study on diabetes observed that GAL contributed to the maintenance of glucose level homeostasis, could increase NO secretion, and inhibit ACh secretion [82]. The contractile activity is determined by the section of the gastrointestinal tract and the species under study [83]. In humans, GAL exhibited the same effectiveness in stimulating the contractile activity of the jejunum as ACh [83]. Increased GAL secretion can be observed when neuronal damage occurs [67,72]. It also exhibits a potential analgesic effect [84]. Under the influence of MP-PET, a shortening of the intestinal villi was observed in the HD group, which can potentially increase GAL expression as a response to tissue damage and the involvement in their regeneration [84]. If we consider GAL as a neuronal damage marker [72], the conclusion can be drawn that significant neuronal damage has occurred under the influence of MP-PET, and that this is the reason for the observed reduction in the populations of CART, VIP, VAChT, and nNOS neurons.

An increase in relation to both GAL and SP is often observed for agents likely to induce inflammation [54,56]. SP is a neuropeptide found in the intestinal neurons, which exhibits modulatory properties, e.g., through its action on the mast cells, macrophages, T and B lymphocytes, the plasmatic cells, and the nerve growth factor (NGF) [70,85]. In the submucous plexus, immune cells can be observed, including the mast cells [86], which can be stimulated to release histamine via, inter alia, SP, or calcitonin gene-related peptide (CGRP), which are among the major pro-inflammatory neuropeptides [2,85,87–89]. SP is involved in neurogenic inflammation, sensory conduction, and nociception [53,57,89,90]. SP, along with neuropeptide Y (NPY) and neurotensin, exhibits antimicrobial activity [3]. SP also pro-inflammatorily stimulates lymphocytes and macrophages, increases the production of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and IL-12 [56,71,88], and is involved in inflammatory processes in the mucosa [53,70]. The inflammation they induce can cause changes in the secretion and intestinal motility patterns [64,87]. SP is often present in cholinergic neurons [65]. Both ACh and SP have a stimulatory effect on the intestinal muscle coat, as well as pro-secretory and vasodilatory effects [53,72,74,88–90]. SP exhibits a high affinity for the NK-1 receptor and a lower affinity for the NK-2 and NK-3 receptors [88]. It is the SP-NK-1 receptor that is involved in neuroimmune reactions in the intestines [88,89]. Through the same receptor, electrolyte and water secretion can also be stimulated in the small intestines and the colons of humans and animals [88–90]. The results obtained may reflect the fact that SP plays a lesser role in the regulation of small intestinal motility and a greater role in neuroprotective, immunological, and nociception processes. MP-PET can directly interact with the intestinal mucosa, which is why an increase in the percentage of SP-positive neurons was observed, especially in the ISP in LD and HD group. A similar response was noted in pigs exposed to *Schistosoma japonicum*, which indicates that more damage to the nerve plexus can be found closer to the intestinal lumen [64]. The aforementioned study observed that the VIP and SP levels varied depending on the severity of inflammation, but there was an increase noted in SP and a decrease in VIP in tissues with mild, moderate, and severe inflammation [64], which corresponds to the results obtained in the present study.

As the ENS is a complex system, a decrease in the percentage of CART-, nNOS-, VAChT-, and VIP-positive neurons and an increase in the percentage of SP- and GAL-positive neurons do not result in a zero-one response because there are other substances (e.g., serotonin, γ-aminobutyric acid (GABA), PACAP, NPY, CGRP, or dopamine), which were not the subject of the experiment described, that affect plasticity of the ENS [1,5,7,52,71,72]. It is noteworthy that no changes, such as a decrease in appetite, behavioural changes, or the occurrence of diarrhoea or growth inhibition (Figure 1), were clinically noted in the

animals concerned throughout the duration of the experiment. It should be noted that the ENS is a complex biological system, and the results obtained represent only a fragment of knowledge on the effects of MP-PET on the domestic pig, and do not present other potential mechanisms that may be responsible for maintaining homeostasis of the entire system (and are likely to go beyond the ENS). It is, therefore, not possible to conclusively determine the ultimate nature of the impact of microplastics on the entire body.

The objective of this study was to identify statistically significant differences in neurotransmitter occurrence between the experimental groups, focusing on *p*-values derived from ANOVA with Scheffe's post hoc test to indicate significant differences without addressing effect magnitudes. Due to the limited sample size, the reliability of effect size measures was potentially compromised, so the analysis prioritises *p*-values to highlight significant findings, with the understanding that future studies with larger samples will provide more robust effect size estimates. Given the complexity and exploratory nature of this investigation, the analysis was streamlined to focus on statistically significant differences, as calculating effect sizes for each neurotransmitter across multiple comparisons would complicate interpretation. Future studies should address these nuances in greater details.

5. Conclusions

The results obtained differ from the results obtained in previous studies [30]. This may be due to the different physicochemical properties of MP-PET and/or the neurotransmitters under study, depending on the section of the gastrointestinal tract. These differences may be due to the fact that MP-PET remains in the digestive tract longer, and is therefore under the influence of intestinal juice. The previously cited studies [42,44] show that digestion may cause changes in physicochemical properties, including the release of nanoparticles or chemical substances. These changes may potentially cause differences in the percentage of CART- and SP-positive neurons in the duodenum and jejunum under the influence of the same factor, MP-PET. Explaining the differences resulting from oral exposure to microplastics between the percentages of CART- and SP-positive neurons in different sections of digestive tract is problematic. The presented research results, according to the authors' current knowledge, described studies are the first research focusing on microplastics, with this methodology and these animals. For this reason, it is difficult to discuss the potential cause of the differences between the percentage of CART and SP positive neurons in the duodenum and jejunum under the influence of MP-PET. Research results show that MP-PET affects miRNAs that target genes associated with lifestyle diseases (insulin resistance, obesity, cardiovascular disease, and cancer) [51]. Therefore, it may be assumed that it may also influence the expression of other genes or specific biochemical pathways [20]. Histological changes in both sections of the small intestine are comparable, but in the duodenum, a greater shortening of the villi was noted. It can therefore be assumed that in the duodenum, the physical aspect of MP-PET (particle size) may be more important, and in the jejunum, the chemical aspect and nanoparticles formed from MP-PET. The similarity in the structure of the gastrointestinal tract of domestic pigs and humans [6] proves that the present study provides valuable knowledge on the potential effects of oral exposure to microplastics on the human gastrointestinal tract, and guides further research into its effects on humans and animals.

Histological changes were more pronounced in the group receiving 1 g/animal/day of MP-PET. The same dose also contributed to the induction of an ENS response by increasing or decreasing the percentage of neurons positive for the tested substances. The only exception were nNOS neurons in OSP, where no changes occurred.

Based on this, it can be concluded that oral exposure to microplastics can affect the ENS function by neurotoxic and pro-inflammatory effects, yet there is a need for further research to determine the mechanism of this process and possible further effects. Based on the results obtained, the conclusion can be drawn to suggest that inflammation, mainly mediated by GAL and SP, is observed in the ENS of the porcine jejunum.

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Institutional Review Board Statement: All procedures were based on the approval given by the Local Ethical Commission (decision No. 10/2020 of 26 February 2020) and in accordance with the Polish law, which sets out the conditions and methods of conducting animal experiments in connection with the Act for the Protection of Animals for Scientific or Educational Purposes of 15 January 2015 (Journal of Laws 2015, no. 266), applicable in the Republic of Poland 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.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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Article

Microplastic and the Enteric Nervous System: Effect of PET Microparticles on Selected Neurotransmitters and Cytokines in the Porcine Ileum

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Abstract: Microplastic is an environmental hazard to which both animals and humans are exposed. Current reports show that it can cause inflammation, including in the gastrointestinal tract. To examine the impact on the ileum, 15 eight-week-old gilts (five individuals/group) were exposed to PET microplastics (7.6 µm–416.9 µm) at a dose of 0.1 g/day or 1 g/day for 28 days. The collected ileum fragments were investigated for the cytokine concentrations (IL-1β, IL-6, IL-8, IL-10, and TNF-α; ELISA test), neuron populations (cocaine and amphetamine-regulated transcript, galanin, neuronal nitric oxide synthase, substance P, vesicular acetylcholine transporter, and vasoactive intestinal peptide; immunofluorescence staining), and morphometric parameters (histological analysis). Under the influence of MP-PET, there was a reduction in the populations of CART- and GAL-positive neurons in the submucosal plexuses and of nNOS-, VAChT-, and VIP-positive neurons in all the plexuses. In contrast, there was an increase in GAL-positive neurons in the myenteric plexus and SP-positive neurons in all the plexuses. The concentrations of IL-1β, IL-6, IL-8, IL-10, and TNF-α did not undergo statistically significant changes under the influence of the low or high dose of MP-PET. The changes in the histological structure exclusively concerned the thinning of the mucosa and the muscularis externa. The results support the thesis that MP-PET is not neutral to the ileal cells.

Keywords: pig; gastrointestinal tract; neurotransmitter; neuropeptide; one health; ingestion; environmental pollution; neuronal plasticity

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1. Introduction

The term "microplastics" is no longer unfamiliar to the public. Microplastics are synthetic particles of primary or secondary origin, transparent or colored, regularly or irregularly shaped [1,2]. When defining these particles, the size range is the biggest problem. According to various authors, in terms of size, microplastics fall into the range of 0.1–1000 µm [3] or 0.1–5000 µm [1,2]. The most common definition provides that microplastics should be regarded as particles with a diameter of 5 mm or smaller [2]. In addition, one can also distinguish nanoplastics, mesoplastics, macroplastics, and megaplastics [3,4], the size range of which can also vary depending on the source. For this reason, the authors believe that an authoritative body should establish a single binding definition to facilitate research and the interpretation of results. This would allow misunderstandings to be avoided in further discussions between researchers. When the phrase "microplastic" is entered into the PubMed, Web of Science, and Scopus databases, an increasing trend in the number of scientific publications on this topic can be observed year after year. The scope of research presented in the literature is wide. The publications on the topic include the results of in vivo and in vitro studies concerning the occurrence, characteristics, hazards, degradation, effects on different ecosystems and living organisms, etc. Materials of different

origins are used, including particles of a certain size/shape or their mixtures and different concentrations/doses based on the unit weight or the number of particles. Despite the upward trend in the ongoing research, there is still a large cognitive gap.

The current study focused on one polymer—polyethylene terephthalate (PET). Plastic packaging is widely used in the food industry because of its convenience (high strength, durability, and low weight). It is estimated that until an alternative to PET packaging is invented, the demand for it will continue to increase [5]. Modern lifestyles, i.e., the popularity of dietary (box) catering, ready-to-eat meals, and "fast fashion", contribute to the environmental release of an increasing number of particles of various sizes, including the above-mentioned polyethylene terephthalate (MP-PET). On the other hand, despite the increased use of plastic products, it should be noted that public awareness of the microplastic problem is growing. As early as 2019, the European Union introduced Directive (EU) 2019/904 of the European Parliament and of the Council of 5 June 2019 on the reduction in the impact of certain plastic products on the environment, and pursuant to it, certain states phased out certain articles, e.g., plastic straws. These measures aim to reduce the amount of particles released into the environment and the volume of plastic waste. It should be noted that plastics, whether on a microscale or a macroscale, can have a harmful effect, but they can also be vectors for heavy metals or antibiotic resistance genes [3].

The One Health Initiative aims to take a multidisciplinary approach to health and demonstrates the links between the environment, animals, and humans [6]. In line with this concept, it was decided to use the pig (*Sus scrofa domestica*) as a research model. Pigs can link environmental microplastics and humans through the food chain. Corte Pause et al. noted that livestock [3] could represent the missing link in the exposure of humans to microplastics. The intake of products of animal origin, e.g., meat or milk, can be a source of microplastics for humans [3]. The presence of nanoparticles and/or microparticles (NMP) has also been demonstrated in vegetables and fruit [7], which can be used for animal feeding but can also be directly consumed by humans. For plants, on the other hand, it is the soil that can be a source of contamination [3]. Another source of NMP can be packaging for animal feed and human food [2,8]. In addition, due to its similarity to humans in terms of physiology, anatomy, immunology, or the genome, the pig is used in biomedical research [9]. This allows one to assess the potential effect of NMP on the entire body and draw conclusions about its effects on humans. The above-mentioned methodology for carrying out the research has both advantages and disadvantages as due to the rules restricting the use of animals for scientific research, the size of study groups is limited. In vivo studies using isolated research models, e.g., specific tissues, are more unpredictable than in vitro studies. They are also inconclusive in nature as it is not always possible to explain the changes observed, or they may not be as significant when considering the organism as a single system striving to maintain homeostasis.

The gastrointestinal tract is constantly exposed to various xenobiotics, and the exposing factors can include food or drink, for example. Xenobiotics and pathophysiological conditions can affect not only the histological structure but also hormone balance, as well as the immune and nervous systems [10]. Xenobiotic-induced changes in one system may result in changes observed in another system, e.g., cytokines may regulate neurotransmission [11], and neurotransmitters/neuropeptides may act as immune modulators [12].

The ileum plays an important role in introducing xenobiotics via the alimentary route. The absorption of nutrients and other substances not absorbed in the duodenum and jejunum occurs in the ileum. In the mucosa and submucosa, Peyer's patches are found, which are one of the components of the gut-associated lymphoid tissue (GALT) [13,14], which can account for up to 70% of the mass of the immune system [14]. Peyer's patches contain M cells capable of collecting an antigen from the intestinal lumen and presenting it to the T and B cells via the dendritic cells. This way, NMP particles can be presented to the immune system cells. Therefore, due to its function, the ileum can also be exposed to MP-PET. In order to compare the effect of MP-PET on cytokine concentration, those that were tested in other experimental systems were selected [4]. Mainly pro-inflammatory

cytokines (IL-1β, IL-8, IL-6, and TNF-α) and IL-10, as a representative of anti-inflammatory cytokines, were selected.

Based on previous studies by the authors, it is known that MP-PET affects the enteric nervous system (ENS) [15,16], which is also referred to as the second brain [17,18]. The term "second brain" stems from the fact that ENS is a component of the autonomic nervous system—a network of neurons and glial cells in the gastrointestinal tract that regulate most functions [17,18]. The ENS comprises a myenteric plexus that mainly regulates the contractile and relaxation functions and the submucous plexus, which affects the regulation of blood flow or the secretory functions [17]. It should be remembered, however, that this is a great oversimplification of the functions of the myenteric and submucous plexus. In pigs and humans, the submucous plexus is divided into the inner (ISP) and the outer (OSP) submucous plexus [19]. Cellular damage in the ENS and its abnormal function can be congenital, e.g., in the course of Hirschsprung's disease [17,18], or secondary, e.g., caused by gastroparesis [17] or inflammation [20]. The results obtained to date [15,16] show that despite using tissues derived from the same animals, the plasticity of the enteric nervous system neurons varies depending on the gastrointestinal tract segment under study. Therefore, the authors decided to carry out a similar study on the ileum, which would provide a comprehensive overview of the effects of microplastics on the small intestine. The selection of cocaine and amphetamine-regulated transcript (CART), galanin (GAL), neuronal nitric oxide synthase (nNOS; nitrergic neuron marker), substance P (SP), vesicular acetylcholine transporter (VAChT; cholinergic neuron marker), and vasoactive intestinal peptide (VIP) was related to the functions performed by these neurotransmitters and neuropeptides in both pathological and physiological conditions. They not only regulate blood flow, secretory functions, and intestinal motility but may also have neuroprotective effects or modulate the immune response [19,20].

In vivo and in vitro studies conducted to date confirm that NMP can exhibit pro-inflammatory effects [4,21], e.g., by inducing oxidative stress [22–24] or cytotoxicity [6]. The effect of NMP may vary depending on the size and the material it is made from [4]. Thus, the authors chose to use particles representing a heterogeneous mixture in the study, even though they were composed of a single material. This was an advantage of this study as it more closely simulated the actual hazard than a study using exclusively one particle size.

This study aimed to determine if and how 28-day exposure to microplastics at doses of 0.1 and 1 g/animal/day administered orally affected the population size of the CART-, GAL-, nNOS-, SP-, VAChT-, and VIP-positive neurons; the concentrations of IL-1β, IL-6, IL-8, IL-10, and TNF-α; and the histological structure of the pig ileum.

2. Results

2.1. Cytokine Concentrations

The concentrations of the cytokines under study are presented in Figure 1A–E. Although differences in the concentrations of the cytokines under study were observed between the groups, statistical analysis did not show them to be significant ($p > 0.05$). In the LD group, the highest concentration was noted for IL-1β (602.83 pg/mg protein ± 51.12) and IL-10 (250.17 pg/mg protein ± 23.73), whereas the lowest was in IL-6 (141.39 pg/mg protein ± 11.90) and TNFα (137.31 pg/mg protein ± 9.12). On the other hand, the high dose increased the concentration of each cytokine in relation to the study group (IL-1β from 487.95 pg/mg protein ± 56.51 to 511.02 pg/mg protein ± 92.93, IL-6 from 186.59 pg/mg protein ± 21.33 to 273.93 pg/mg protein ± 55.50, IL-8 from 438.18 pg/mg protein ± 31.44 to 615.34 pg/mg protein ± 143.42, IL-10 from 193.75 pg/mg protein ± 19.88 to 253.57 pg/mg protein ± 40.49, and TNFα from 143.77 pg/mg protein ± 17.59 to 249.99 pg/mg protein ± 52.46).

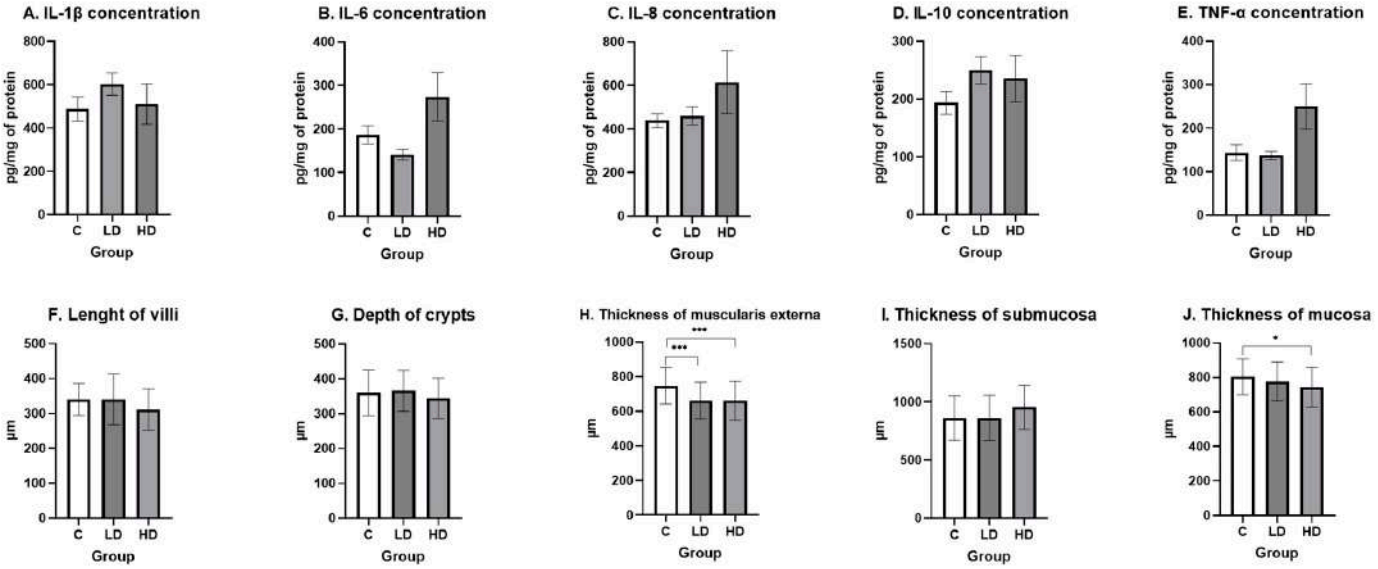


Figure 1. Concentrations of selected cytokines in the ileal wall (**A**–**E**) and the morphometric indices (**F**–**J**) of the ileal wall. The cytokine concentration results are presented as a mean (pg/mg protein) ± SEM. The morphometric measurement results are presented as a mean (µm) ± SD. C—control group; LD—low-dose group; HD—high-dose group. * $p < 0.05$, *** $p < 0.001$ indicate statistically significant differences.

2.2. Histological Study

Under the influence of the high dose of MP-PET, there was a thinning of the thickness of the mucosa (from 803.63 µm ± 104.70 to 743.39 µm ± 114.73; $p < 0.05$) and the thickness of muscularis externa (from 746.36 µm ±106.04 to 660.30 µm ±112.69; $p < 0.001$). The thickness of the muscularis externa (661.73 µm ± 105.25; $p < 0.001$) was also lower, as compared with the control group, in animals from the low-dose MP-PET group. As for the other indices, i.e., the length of the villi, the crypt depth, and the thickness of the submucosa, no statistically significant differences were noted ($p > 0.05$). The length of the intestinal villi ranged from 310.66 µm ± 59.00 to 340.10 µm ± 46.05, the crypt depth from 344.37 µm ± 57.32 to 365.70 µm ± 59.13, and the thickness of the submucosa from 860.01 µm ± 188.99 to 953.56 µm ± 191.43 (Figure 1F–J).

2.3. Immunofluorescence Staining

2.3.1. Cocaine and Amphetamine Regulated Transcript

CART-positive neurons were among the least numerous populations of the neurotransmitters under study. The myenteric plexus neurons were the only neurons unaffected by the low or high dose of MP-PET. Their percentage ranged between 5.67% ± 0.99 (C) and 4.25% ± 0.51 (HD). However, both doses reduced the CART-positive neuron population size (C 5.23% ± 0.59), with the low dose doing so to a lesser extent (3.67% ± 1.23; $p < 0.05$) than the high dose (2.36% ± 0.83; $p < 0.001$) in OSP. In ISP, however, the reduction in the population size of neurons (C 8.62% ± 0.85) was equally significant under the influence of both MP-PET doses (LD: 3.81% ± 0.94, and HD: 1.78% ± 0.47; $p < 0.001$). The results and microscopic images are presented in the Figure 2.

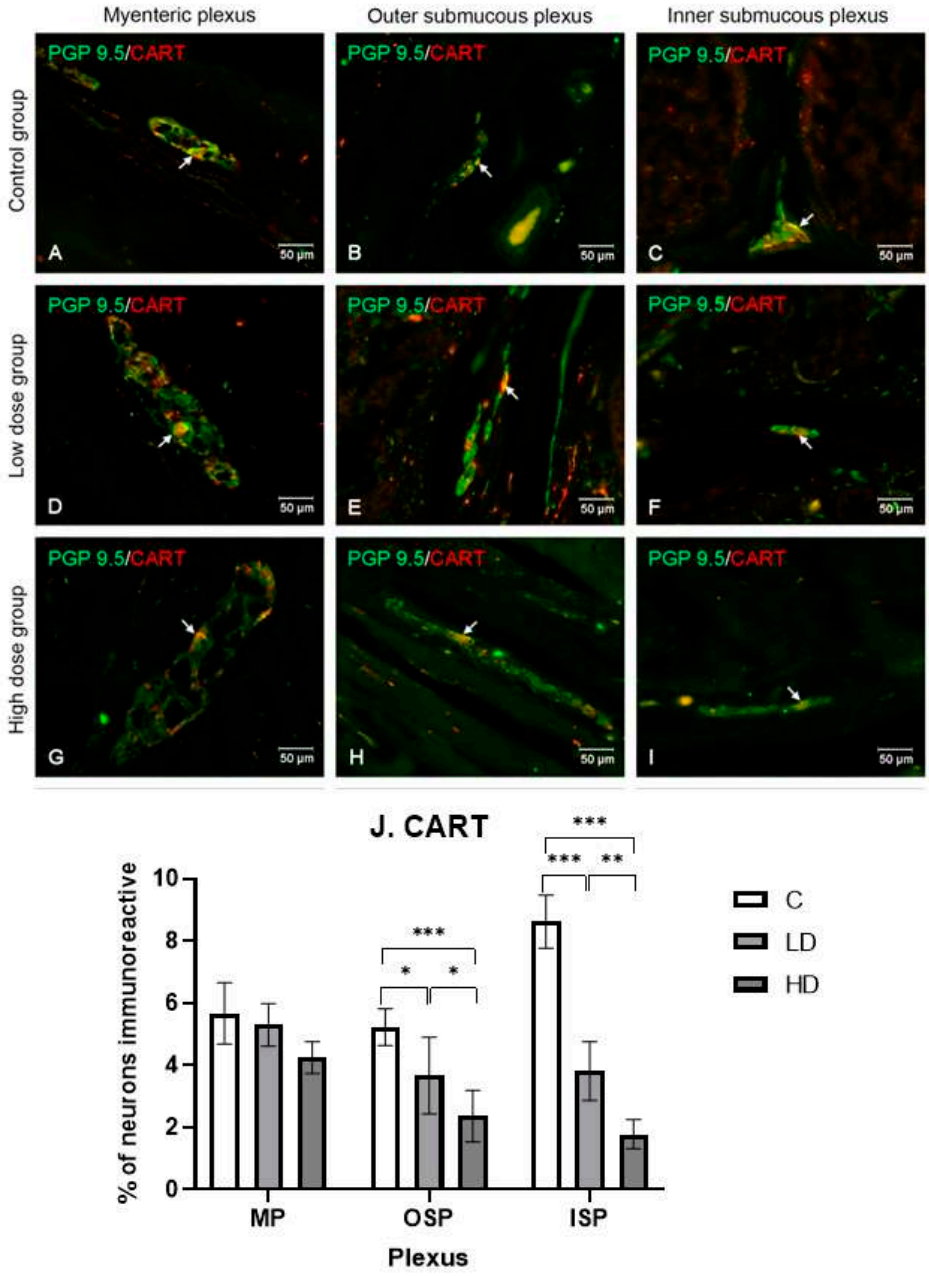


Figure 2. Distribution pattern for neurons immunoreactive toward protein gene product 9.5 and CART. (**A**,**D**,**G**) Myenteric plexus, (**B**,**E**,**H**) outer submucous plexus, and (**C**,**F**,**I**) inner submucous plexus in the C group (**A**–**C**), LD (**D**–**F**), and HD (**G**–**I**). The photographs were created by overlaying two colors (green for PGP 9.5 and red for CART). The arrows indicate cells immunoreactive toward the test substance. (**J**) The percentage of neurons immunoreactive toward CART, expressed as a mean (%) ± SEM. C—control group; LD—low-dose group; HD—high-dose group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate statistically significant differences.

2.3.2. Galanin

GAL-positive neurons in control animals accounted for 4.86% ± 0.61 (MP), 15.84% ± 0.90 (OSP), and 24.18% ± 1.11 (ISP). Under the influence of the high dose, statistically significant differences ($p < 0.001$) were noted in each plexus under study, namely, in MP (12.13% ± 1.18), in OSP (8.68% ± 1.40), and in ISP (11.19% ± 1.31). The low dose in MP contributed to an increase in the GAL-positive neuron percentage to 8.86% ± 0.92 ($p < 0.001$) and in OSP to a decrease to 12.66% ± 0.37 ($p < 0.01$). The lack of differences

was demonstrated in ISP (25.10% ± 1.73, $p > 0.05$). The results and microscopic images are presented in the Figure 3.

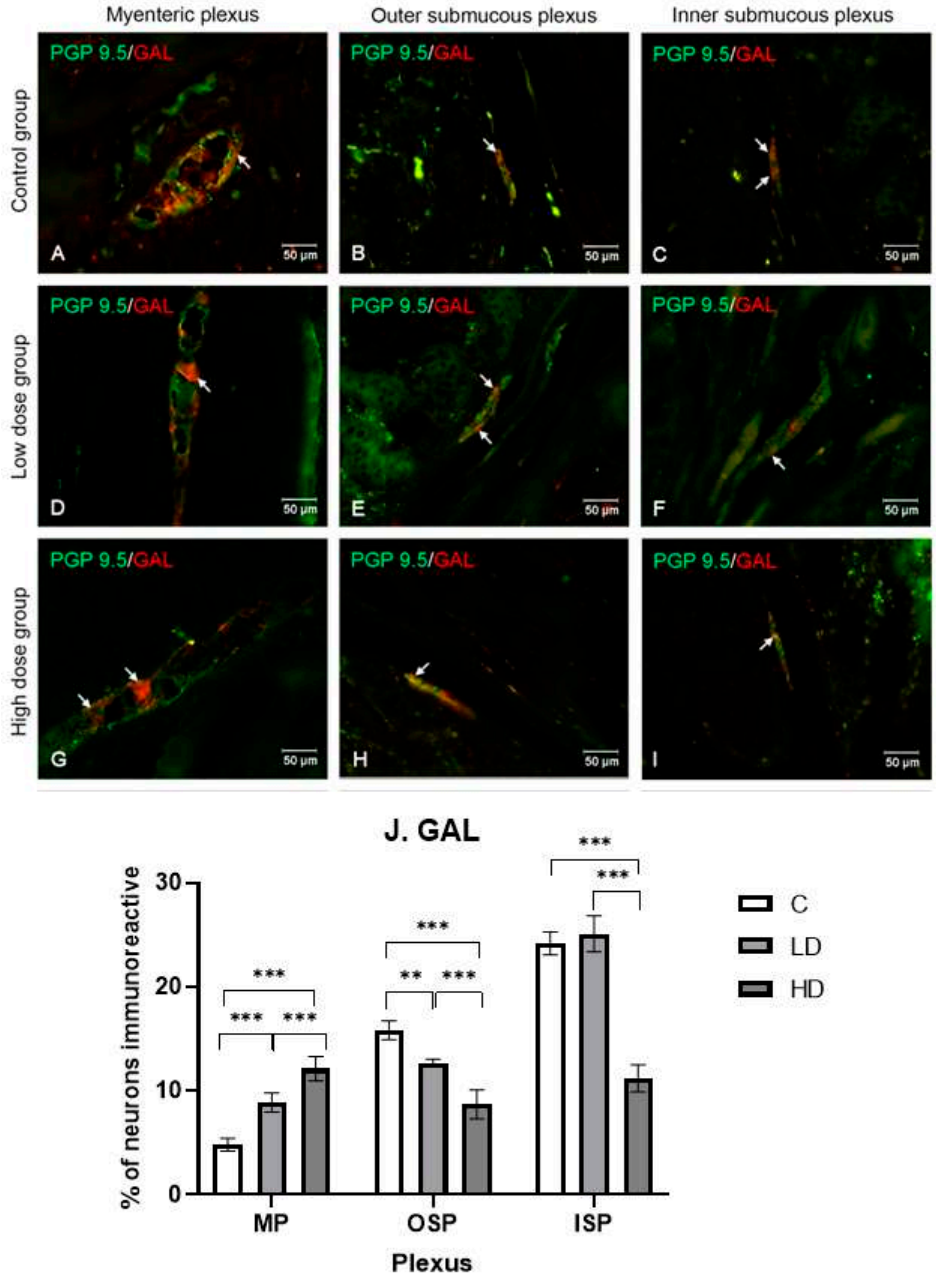


Figure 3. Distribution pattern for neurons immunoreactive toward protein gene product 9.5 and GAL. (**A**,**D**,**G**) Myenteric plexus, (**B**,**E**,**H**) outer submucous plexus, and (**C**,**F**,**I**) inner submucous plexus in the C group (**A**–**C**), LD (**D**–**F**), and HD (**G**–**I**). The photographs were created by overlaying two colors (green for PGP 9.5 and red for GAL). The arrows indicate cells immunoreactive toward the test substance. (**J**) The percentage of neurons immunoreactive toward GAL, expressed as a mean (%) ± SEM. C—control group; LD—low-dose group; HD—high-dose group ** $p < 0.01$, *** $p < 0.001$ indicate statistically significant differences.

2.3.3. Neuronal Nitric Oxide Synthase

In the control group, the percentage of nNOS-positive neurons accounted for 25.74% ± 1.53 (MP), 11.54% ± 1.51 (LD), and 13.47% ± 1.79 (HD). The high dose of MP-PET contributed to a decrease in the nNOS-positive neuron population in all the plexuses, in MP to 12.78% ± 1.06 ($p < 0.001$), in OSP to 8.75% ± 1.00 ($p < 0.001$), and in ISP to

6.87% ± 1.52 ($p < 0.001$). However, the low dose of MP-PET only resulted in a difference in MP, 17.90% ± 1.05 ($p < 0001$). In OSP (11.03% ± 0.88) and ISP (13.59% ± 1.86), no statistically significant differences ($p > 0.05$) under the influence of LD MP-PET were noted. The results and microscopic images are presented in the Figure 4.

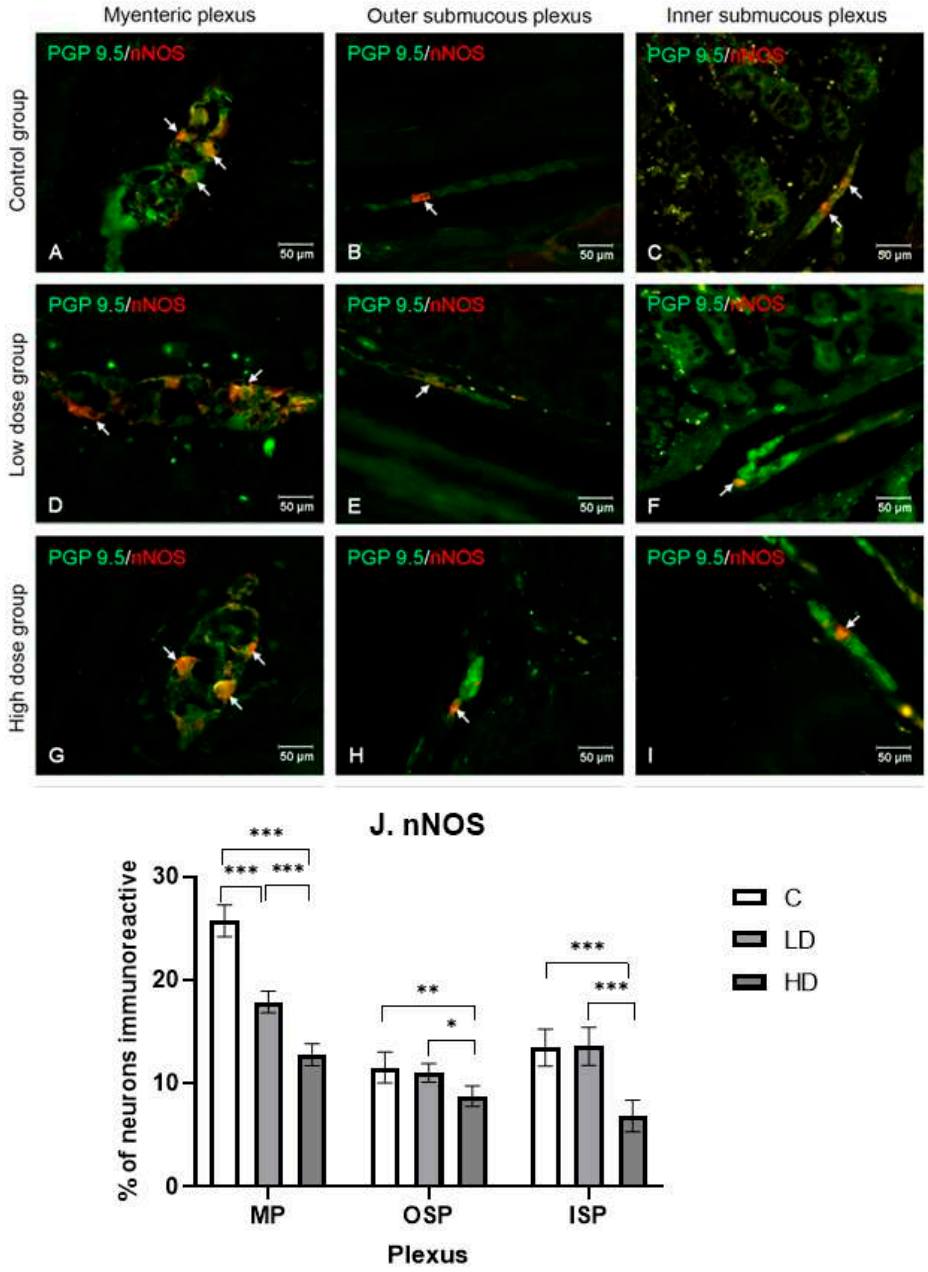


Figure 4. Distribution pattern for neurons immunoreactive toward protein gene product 9.5 and nNOS. (**A**,**D**,**G**) Myenteric plexus, (**B**,**E**,**H**) outer submucous plexus, and (**C**,**F**,**I**) inner submucous plexus in the C group (**A**–**C**), LD (**D**–**F**), and HD (**G**–**I**). The photographs were created by overlaying two colors (green for PGP 9.5 and red for nNOS). The arrows indicate cells immunoreactive toward the test substance. (**J**) The percentage of neurons immunoreactive toward nNOS, expressed as a mean (%) ± SEM. C—control group; LD—low-dose group; HD—high-dose group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate statistically significant differences.

2.3.4. Substance P

Under the influence of the low and high doses of MP-PET, the percentage of SP-positive neurons increased in all the plexuses ($p < 0.001$). In the MP, in the C group, the

percentage was 2.68% ± 0.39, 6.99% ± 1.12 in the LD group, and 8.94% ± 1.62 in the HD group. In OSP, in the C group, 19.07% ± 1.91 SP-positive neurons were noted; in OSP, 24.79% ± 2.24; and in ISP, 24.13% ± 1.59. Similarly, changes occurred in ISP, i.e., in the C group, 21.90% ± 1.68; in OSP, 27.61% ± 2.41; and in ISP, 31.96% ± 1.11. The results and microscopic images are presented in the Figure 5.

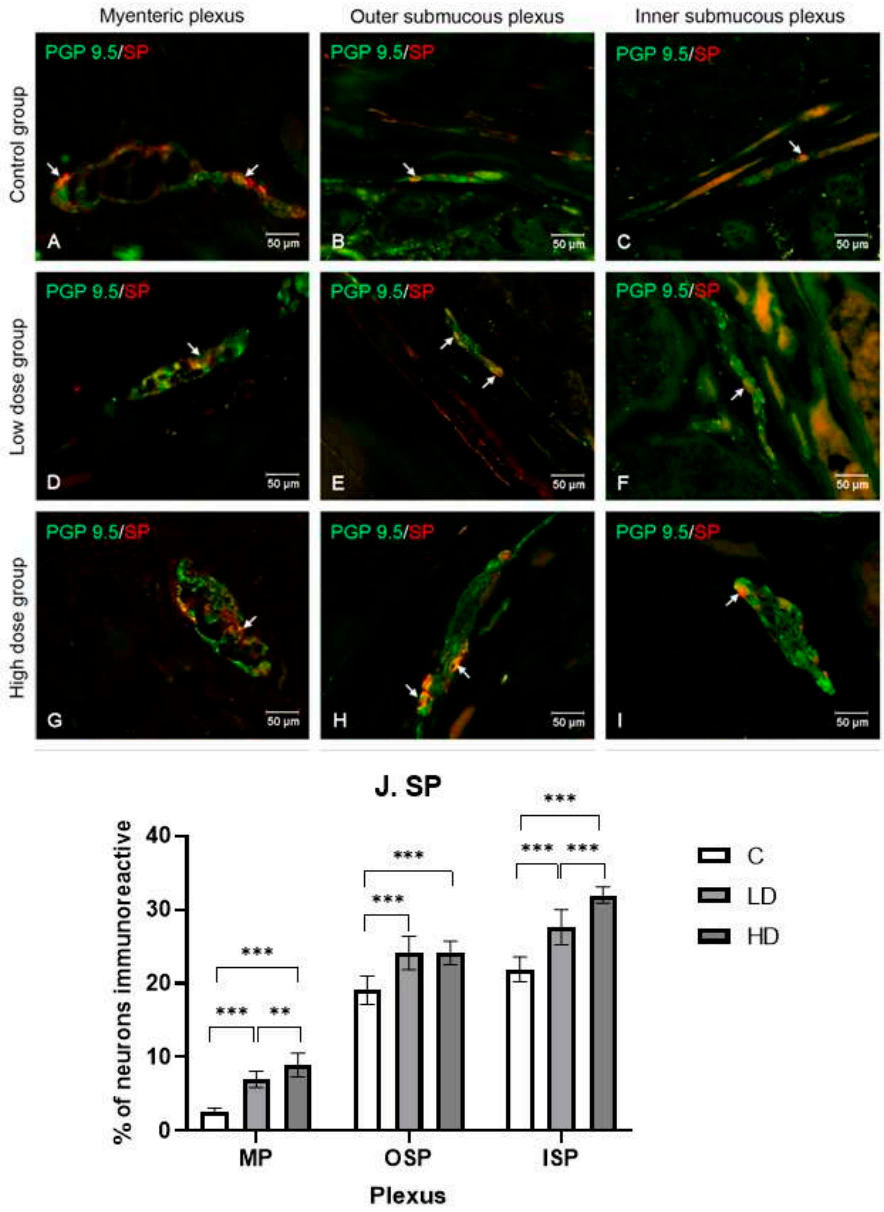


Figure 5. Distribution pattern for neurons immunoreactive toward protein gene product 9.5 and SP. (**A**,**D**,**G**) Myenteric plexus, (**B**,**E**,**H**) outer submucous plexus, and (**C**,**F**,**I**) inner submucous plexus in the C group (**A**–**C**), LD (**D**–**F**), and HD (**G**–**I**). The photographs were created by overlaying two colors (green for PGP 9.5 and red for SP). The arrows indicate cells immunoreactive toward the test substance. (**J**) The percentage of neurons immunoreactive toward SP, expressed as a mean (%) ± SEM. C—control group; LD—low-dose group; HD—high-dose group. ** $p < 0.01$, *** $p < 0.001$ indicate statistically significant differences.

2.3.5. Vesicular Acetylcholine Transporter

The VAChT-positive neurons were the most abundant in the MP (23.09% ± 0.84 (the C group)), followed by ISP (20.92% ± 1.68 (the C group)). They were the least abundant in OSP (16.77% ± 2.14 (the C group)). The low dose of MP-PET reduced the positive neuron

percentage to 19.67% ± 0.87 in MP and to 13.38% ± 0.56 in OSP ($p < 0.01$). No statistically significant changes were noted in ISP (20.32% ± 1.40, $p > 0.05$). Under the influence of the high dose of MP-PET, the greatest differences were observed in MP (9.30% ± 2.04) and ISP (15.32% ± 1.85) ($p < 0.001$). In the OSP, the differences were less pronounced than the previously mentioned plexuses (13.38% ± 0.56) ($p < 0.01$). The results and microscopic images are presented in the Figure 6.

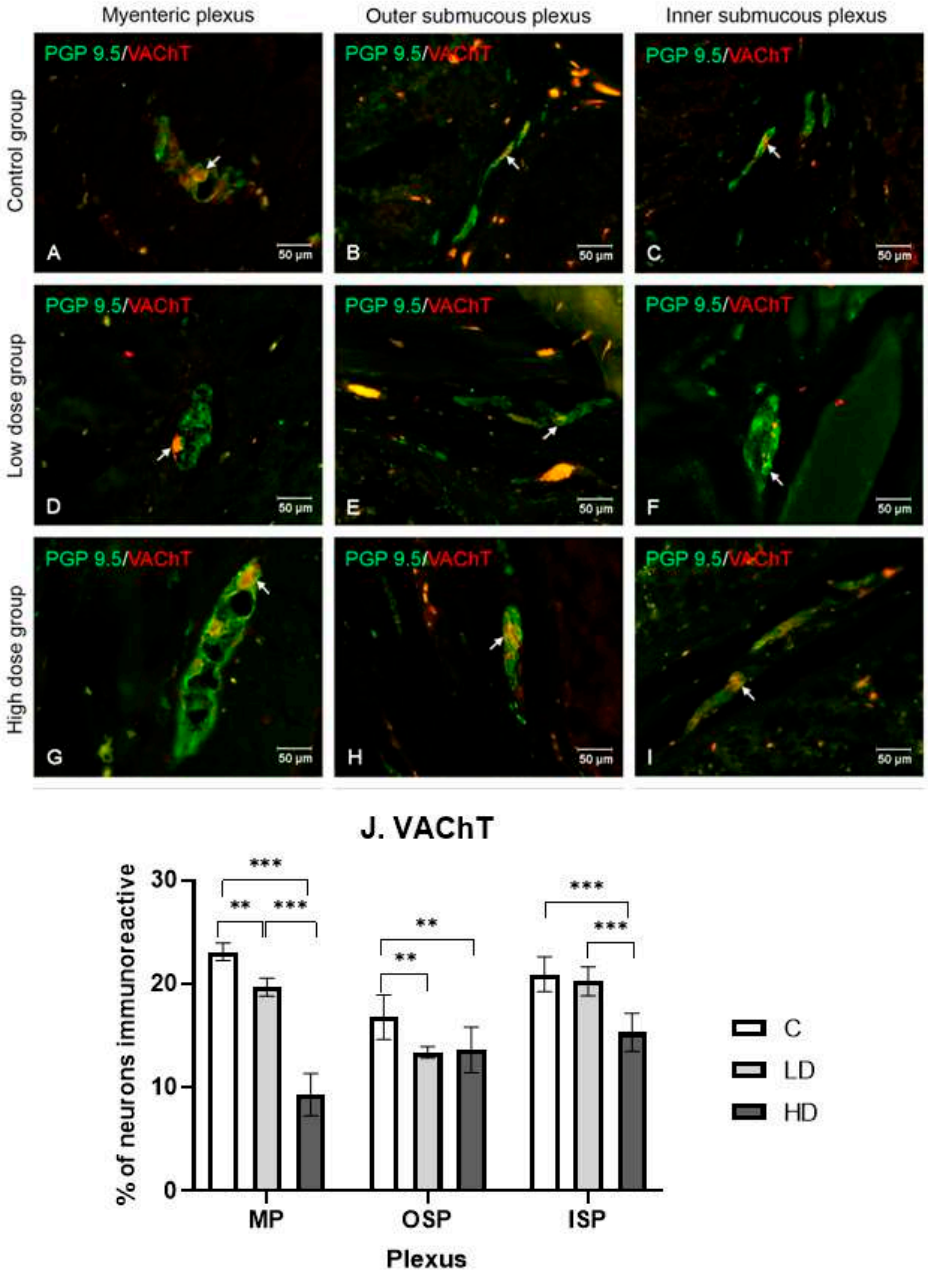


Figure 6. Distribution pattern for neurons immunoreactive toward protein gene product 9.5 and VAChT. (**A**,**D**,**G**) Myenteric plexus, (**B**,**E**,**H**) outer submucous plexus, and (**C**,**F**,**I**) inner submucous plexus in the C group (**A**–**C**), LD (**D**–**F**), and HD (**G**–**I**). The photographs were created by overlaying two colors (green for PGP 9.5 and red for VAChT). The arrows indicate cells immunoreactive toward the test substance. (**J**) The percentage of neurons immunoreactive toward VAChT, expressed as a mean (%) ± SEM. C—control group; LD—low-dose group; HD—high-dose group. ** $p < 0.01$, *** $p < 0.001$ indicate statistically significant differences.

2.3.6. Vasoactive Intestinal Peptide

In the control group, 15.57% ± 0.78 (MP), 25.21% ± 1.53 (LD), and 27.38% ± 1.47 VIP-positive neurons were noted. Both the low and the high doses of MP-PET resulted in statistically significant differences at $p < 0.001$ in all plexuses. In MP, there was a reduction in the neurons immunoreactive toward VIP, to 7.59% ± 0.71 (LD) and 6.17% ± 0.51 (HD). In ISP, however, there was a reduction to 17.33% ± 1.16 (LD) and 18.10% ± 1.54 (HD), and in OSP, there was a reduction to 13.86% ±1.34 (LD) and 13.85% ±1.42 (HD). The results and microscopic images are presented in the Figure 7.

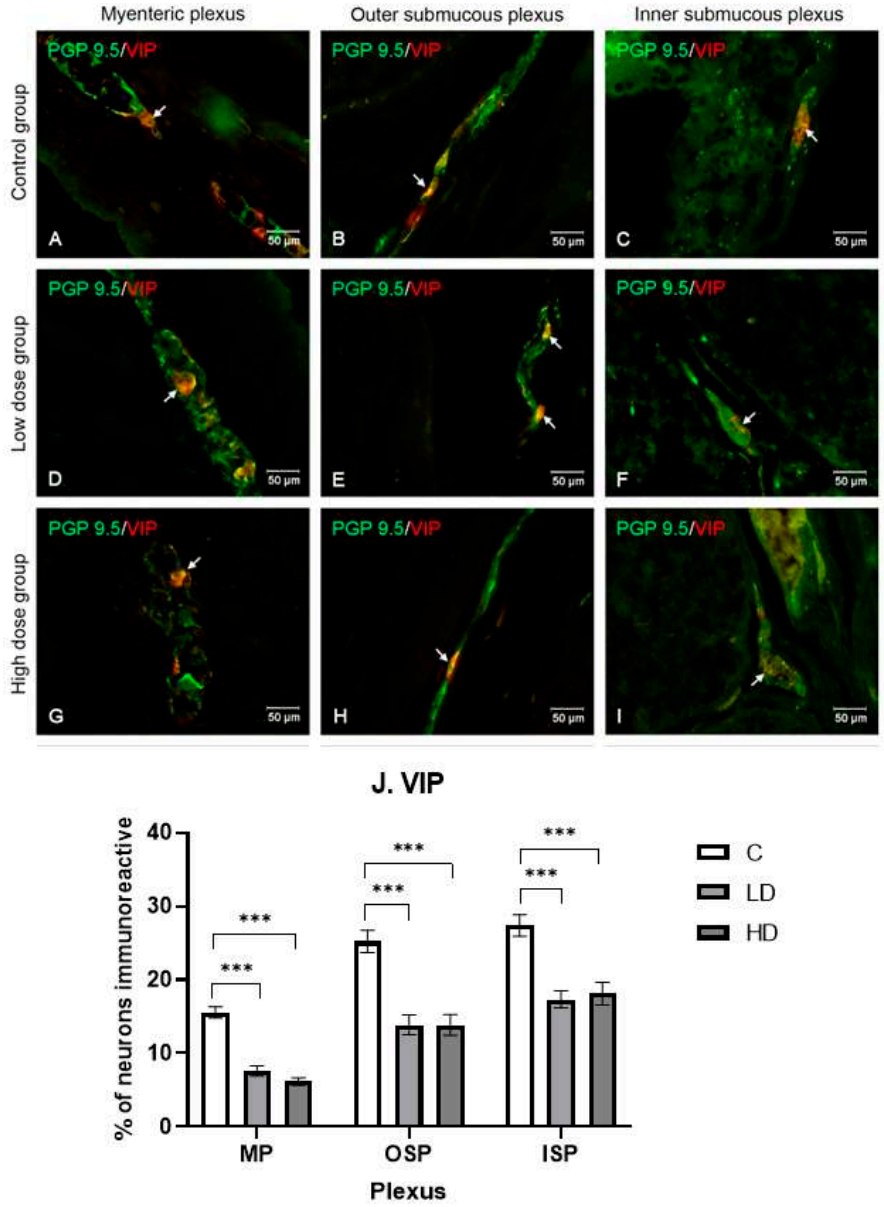


Figure 7. Distribution pattern for neurons immunoreactive toward protein gene product 9.5 and VIP. (**A**,**D**,**G**) Myenteric plexus, (**B**,**E**,**H**) outer submucous plexus, and (**C**,**F**,**I**) inner submucous plexus in the C group (**A**–**C**), LD (**D**–**F**), and HD (**G**–**I**). The photographs were created by overlaying two colors (green for PGP 9.5 and red for VIP). The arrows indicate cells immunoreactive toward the test substance. (**J**) The percentage of neurons immunoreactive toward VIP, expressed as a mean (%) ± SEM. C—control group; LD—low-dose group; HD—high-dose group. *** $p < 0.001$ indicate statistically significant differences.

3. Discussion

Reports on the effects of NMP on pigs are currently very scarce. They mainly focus on the identification of NMP in feces [25] or tissues [26] or the use of porcine tissues in in vitro models [27–29]. In contrast to the fairly well-understood effect on mice, rats, or aquatic animals, the effect on pigs still appears enigmatic. In addition, only recently have more studies reported using PET in research. For these reasons, it is difficult to determine the exact mechanism behind the occurrence of the observed changes. Most studies on the effect of NMP report changes in the intestinal barrier integrity [23] and an increase in pro-inflammatory cytokines [4,23] or an increase in the oxidative stress marker levels [4,23,28,29]. They also report higher toxicities for smaller particles [30]. In addition to the authors' articles, only single studies using pigs as the in vivo research model are available. However, they use different types of plastics, animal ages, and doses. Studies using polystyrene microplastics have shown that they contribute to diarrhea, impaired angiogenesis in the gut, and impaired meat quality [31,32]. The difference concerned the age of the animals used, the dose, and the particle size. According to the research, the piglets received an average of approximately 632.25 mg and 1198.5 mg of microplastics when reaching their final body weight. The current study used a daily dose rather than a dose per kg body weight since the authors believed this more closely represented environmental exposure [33]. Since compared with the above-mentioned studies, the daily dose may appear to be a much milder dose (the low dose is lower by approximately 250 times, and the high dose is lower by approximately 50 times), it is possible not to observe such significant changes as those observed in the above-mentioned studies [31,32]. However, according to the results, even such low doses can induce changes in the ENS.

Given the reported pro-inflammatory effect of NMP, it might be expected that the percentage of GAL-, VIP-, nNOS-, SP-, or CART-positive neurons would be increased after oral exposure, which is linked to the neuroprotective properties of these neurotransmitters and neuropeptides [34–37]. The available results, however, show that the population of these neurons decreased, except for the GAL-positive neurons in MP and SP-positive neurons in all plexuses. Similarly, one could suspect that the pro-inflammatory cytokine levels should increase. Other studies indicate that NMP can increase pro-inflammatory cytokine levels [23] but may not induce any statistically significant changes [4,38]. It should be noted, however, that PET was not used in any of the cases cited. The results obtained by the authors show certain upward trends: it can be observed that with an increase in the MP-PET dose, the concentrations of TNFα, IL-6, and IL-8 increase, with the p value, however, remaining at a level of >0.05.

It can also be assumed that MP-PET does not affect all the neurotransmitters and neuropeptides in the same way. The lack of differences for CART in MP and nNOS between the C group and the LD group in OSP and ISP may either be indicative of this fact or provide evidence of changes that are only just developing, as is the case of cytokine concentrations under the influence of the mycotoxin zearalenone, where differences in cytokine levels are shown in the ileum of pigs of similar body weight [39]. Analysis of the results shows that the cytokine levels may vary depending on the day of testing. When comparing the IL-1β concentration on days 14, 28, and 42, one can observe that on day 28, there was a decrease in the concentration, as compared with day 14, and on day 42, an increase took place again [39]. The same was true for IL-8 or TNFα [39]. Further research is needed to confirm whether the dose of MP-PET chosen by the authors would contribute to significant changes but under longer exposure or whether the doses chosen are incapable of producing a noticeable effect. Given the differences observed in the percentage of neurons, the authors believe that the 4-week exposure may have contributed to neuroplasticity changes but without significant immune system stimulation. Alternatively, it is also possible that the 28-day exposure contributed to a faster adaptation of the immune system and a return to homeostasis than ENS and hence the observed discrepancies between the studied plexuses or other sections of the small intestine.

Microplastics have been shown to contribute to changes in the expression and activity of such neurotransmitters as dopamine, glutamate, serotonin, gamma-aminobutyric acid, and acetylcholine [21,40]. The neurotoxicity of NMP has been linked to the effect on neurotransmission, e.g., by affecting the expression of the genes involved [24,40] or reducing nerve cells [41]. MP-PET appears to affect VAChT-, nNOS-, and VIP-positive neurons equally, irrespective of the small intestine segment. The differences presented in this study not only are due to the potential changes in the physical and/or chemical structure of microplastic but also may confirm that the function of neurotransmitters is determined not only by the segment of the gastrointestinal tract but also by the plexus.

The level of expression of neurotransmitters and neuropeptides can also be affected by time and the tissue fragment examined, even of the same gastrointestinal tract segment. Studies showing the colocalization of CART- and/or GAL-positive neurons in a stomach with adenocarcinoma have demonstrated that the number of these fibers increased in the neoplastic area compared with the area showing no abnormal cells [42]. On the other hand, a study on the seabream (*Sparus aurata*) brains observed that changes in the levels of brain monoaminergic neurotransmitters after a 30-day period of microplastic detoxification showed a tendency to normalization [43]. Considering that the likely exposure to NMP in daily life is not the same every day, this provides an opportunity for ENS to adapt smoothly to this xenobiotic.

A study comparing PET and polyethylene showed that PET may exhibit potentially greater toxic properties, e.g., due to its superior oxidative properties and its capability of dissociating guanine–cytosine nucleoside pairs, which may affect DNA replication [44]. Additionally, nanoplastics were demonstrated to exhibit the capability of affecting miRNA [45]. The differences in the percentage of neurons positive to the test substances may be due to abnormal expression of miRNA, for example, which may regulate the cell cycle, growth, development, or apoptosis of cells in ENS [46]. Polystyrene microplastics are known to disrupt intestinal angiogenesis, specifically by destabilizing the mRNA of angiogenic factors [31]. In addition, it was also demonstrated that nanoparticles can contribute to abnormalities in the protein folding process [47] or damage the synapses [48]. Abnormalities may also concern neurotransmitter receptors [49]. Polystyrene nanoplastics have been noted to exhibit different toxicities depending on the cells/tissues under study [22]. Changes in the plasticity of enteric nervous system neurons and neurotransmission [50], changes in protein folding [47], and changes in the gut microflora [51] may be responsible for the development of neurodegenerative diseases. NMP contaminants could potentially be a factor that promotes the development of neurodegenerative or neurodevelopmental diseases [48], but this requires further research. Under the influence of digestion, the microplastic's physicochemical properties may change, which could be the reason for the observed differences in different segments of the small intestine or the entire gastrointestinal tract.

Changes in the thickness of the muscularis externa and the population of VAChT-, VIP-, and CART-positive neurons may indicate changes occurring in the motor activity of the ileum under the influence of MP-PET. Interestingly, the thinning and the smaller population of VAChT-positive neurons may indicate reduced contractility as acetylcholine is one of the main factors stimulating smooth muscle contraction, similar to SP [52]. When exposed to polystyrene, acetylcholine production in mice is reduced [53]. On the other hand, VIP and NO are the main factors causing the relaxation of the muscle layer [52], and these are reduced under the influence of MP-PET. Therefore, this should result in increased intestinal contractility, especially with an increase in the percentage of SP-positive neurons. However, other mechanisms involved are not the subjects of the study presented here. During the experiment, no clinical changes in motility, manifested as constipation or diarrhea, were noted, but smooth muscle contractility tests would be required to confirm changes in intestinal motility. In addition, there are reports that CART can promote the survival of neurons of the muscularis mucosae and/or smooth muscles [54]. In the course of Hirschsprung disease, the population size of CART-positive neurons decreases [55], similar to the present study, where the same was noted in the submucosal plexus area.

Interestingly, a downward trend in the CART-positive neuron population size was noted in the MP, but this was not statistically significant ($p > 0.05$).

Wang et al. studied the effects of low-density polyethylene microplastics, or their oxidized form, on the microbiome–gut–brain axis. They demonstrated that acetylcholine levels were significantly reduced in the cerebral cortex and the hippocampus, and acetylcholinesterase activity was significantly increased, indicating abnormalities in neurotransmission and that a similar mechanism underlies Alzheimer's disease [50]. An additional aspect that makes the microplastic-induced changes similar to neurodegenerative diseases, in addition to changes in the neurotransmitter levels, includes changes in the histological picture, changes in the intestinal barrier integrity, or the stimulation of the immune system [21]. The presented study also suggests that microplastics may be a factor that will promote the occurrence of neurodegenerative diseases, e.g., Alzheimer's disease.

If further research is conducted into the effects of NMP on neurons and neuronally active substances, and if results similar to those presented in this article are demonstrated, the use of endogenous VIP, for example, may be part of counteracting the negative effects of exposure to NMP. This is all the more important because VIP can affect the intestines and the entire body [56].

Limitations of the presented research concern the small size of the study group, which, however, is related to the reduction in the use of animals in in vivo research. In addition, the research model is based on the use of animals of the same breed originating from the same farm. It was shown that the origin and breed of animals can influence the level of neurotransmitters [57] and the gut microbiome [58]. The study results presented here only show the potential effect of MP-PET without investigating the mechanism behind them. Additionally, in the analysis of the results, p values were prioritized as a measure of statistical significance. Due to the exploratory nature of the studies and limitations related to sample sizes ($n = 5$), the effect magnitudes of the tests used may be potentially compromised.

The results support the thesis that MP-PET is not neutral to the ileal cells. Even such low doses are capable of inducing an ENS response in the form of changes in the population of neurons immunoreactive toward CART, GAL, nNOS, SP, VAChT, and VIP. Under the influence of MP-PET, there is a reduction in the populations of CART- and GAL-positive neurons in the submucosal plexuses and of nNOS-, VAChT-, and VIP-positive neurons in all the plexuses. In contrast, there is an increase in GAL-positive neurons in the myenteric plexus and SP-positive neurons in all the plexuses. The concentrations of IL-1β, IL-6, IL-8, IL-10, and TNF-α did not undergo statistically significant changes under the influence of the low or high dose of MP-PET. However, the changes in the histological structure exclusively concerned the thinning of the mucosa and the muscularis externa. Undoubtedly, further research focusing on the effects of NMP in the gastrointestinal tract should be carried out as it may contribute to building a strategy for coping with NMP in humans and animals.

4. Materials and Methods

The procedure for carrying out the experiment, performing double immunofluorescence staining, and conducting histological examinations was followed according to the protocol and using the reagents indicated in publications by Gałęcka et al. [15,16].

4.1. Microplastic

The characterization of PET (Cat. No ES306031/1, Goodfellow Cambridge Ltd., Cambridge, UK) in powder form was carried out using a Mastersizer 2000 analyzer (Malvern Instruments Ltd., Worcestershire, UK) and a Phenom ProX G6 scanning microscope (ThermoFisher Scientific, Waltham, MA, USA). This analysis found that 10% of the particles under study had a diameter of less than 51.6 µm, 50% had a diameter of less than 124.6 µm, and 90% had a diameter of less than 237.0 µm. The test sample contained particles with diameters ranging from 7.6 µm to 416.9 µm, with the predominant proportion of particles with a diameter of 158.5 µm. Analysis of the microscopic image showed that the

used powder was a mixture of particles of different surface areas, sizes, and shapes. The characteristics of the used particles are provided in previous publications [16].

Two doses were applied: a low dose (LD) of 0.1 g/day and a high dose (HD) of 1 g/day. They were administered daily in gelatine capsules before the morning feeding. The LD dose represented a realistic exposure to microplastics (according to Senathirajah et al., 0.1–5 g microplastics/week/person [33], translating into 0.01–0.71 g microplastics/day/person). In addition, when establishing the low dose, the study by Deng et al. was used as a guide [59]. Based on this study, the dose was adjusted to the weight of the pigs (approximately 20 kg b.w.). The HD dose, on the other hand, was calculated based on the decimal algorithm (for $\log(0,1) = -1$; for $\log(1) = 0$) and was a dose ten times higher than the LD dose.

4.2. Animals

All procedures were based on the approval given by the Local Ethical Commission (decision no. 10/2020 of 26 February 2020) and in accordance with the Polish law, which sets out the conditions and methods of conducting animal experiments in connection with the Act of 15 January 2015 on the Protection of Animals for Scientific or Educational Purposes of (Journal of Laws 2015, No 266), applicable in the Republic of Poland, 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 experiment used 15 sexually immature gilts (*Sus scrofa domestica*) of the Pietrain X Duroc breed, from a farm in Lubawa (Poland), aged approximately 8 weeks, which were divided into three study groups ($n = 5$). The assignment of animals to research groups was performed using a computer random order generator. The first group (C) was administered empty gelatine capsules, the second group received the low microplastic dose (LD), and the third group received the high microplastic dose (HD). Animal handling started with animals from the control group, then LD and HD, and the person taking care of the animals was not informed about the animals' affiliation to the research group. After a one-week acclimatization period, the pigs were subjected to a 28-day oral exposure to microplastics. Throughout the period, they were kept in three pens containing five animals each, with no contact between individuals from different groups. Room temperature was maintained at 21 ± 1 °C and relative humidity at $57.5 \pm 2.5\%$. A 12 h light/dark cycle was used. Feed was administered twice a day and water ad libitum. Stainless steel feeders were used for animal feeding and watering. The animals were kept in the premises of the Faculty of Veterinary Medicine of the University of Warmia and Mazury in Olsztyn

After 28 days, all the animals were euthanized. The animals were administered intramuscularly with atropine (0.05 mg/kg i.m., Polfa, Warsaw, Poland), xylazine (3 mg/kg, i.m., Vet-Agro, Lublin, Poland), and ketamine (6 mg/kg, i.m., Vetoquinol Bio-wet, Gorzów Wlkp., Poland) [60]. When their unconsciousness was confirmed, their vital functions were arrested by an overdose of sodium pentobarbital (0.6 mL/kg i.v., Biowet, Puławy, Poland). Tissue collection started once the absence of corneal reflex, pulse, and respiratory and cardiac murmurs was confirmed. Segments of the ilium, located 2 cm upstream of the ileocecal valve, were collected for further testing.

4.3. Determination of Cytokine Concentrations

Immediately after the collection, the intestinal segments were frozen in liquid nitrogen and kept at −80 °C until the performance of analyses. Each sample (1 g) was homogenized using an extraction buffer consisting of PBS (Cat. No. P4417, Sigma-Aldrich, Saint Louis, MO, USA), protease inhibitors (cOmplete ULTRA Tablets, Mini, EASYpack Protease Inhibitor Cocktail, Cat. No. 05892970001, Sigma-Aldrich, St. Louis, MO, USA), and a homogenizer (Homogenizer T10 Basic Ultra-Turrax IKA Poland Sp. z o.o., Warsaw, Poland). It was then centrifuged at $5000\times g$ for 60 min (Eppendorf 5804R centrifuge, Eppendorf Netherlands B.V., Nijmegen, Netherlands), and the resulting supernatant provided the materials for further analysis using commercial ELISA kits (Table 1) in accordance with the manufacturer's instructions. Previously, the protein concentration was determined using

Qubit Protein Assay (Cat. No. Q33211, ThermoFisher Scientific, Waltham, MA, USA) and Qubit 2.0 Fluorometer (Cat. No. Q32866, ThermoFisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's instructions. Absorbance measurements were taken using a Multiskan FC Microplate Photometer (ThermoFisher Scientific, Waltham, MA, USA) in two replications. The cytokine concentration in the homogenate samples was compared with the protein content.

Table 1. List of the kits used for ELISA testing.

Antigen	ELISA Kit	Catalog Number	Supplier	Assay Range pg/mL
Tumor necrosis factor α	Porcine TNF-alpha ELISA Kit	orb566582	Biorbyt, Cambridge, United Kingdom	32.25–2000
Interleukin 1β	Porcine IL-1beta ELISA Kit	orb566534		62.5–4000
Interleukin 6	Porcine IL-6 ELISA Kit	orb566538		31.25–2000
Interleukin 8	Porcine IL-8 ELISA Kit	orb566594		15.625–1000
Interleukin 10	Porcine IL-10 ELISA Kit	orb566526		32.25–2000

4.4. Histological Study

The tissue samples were fixed in 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4) for 48 h, dehydrated in ethanol (TP 1020, Leica, Wetzlar, Germany), and embedded in paraffin (EG1150, Leica, Wetzlar, Germany). The 4 µm thick sections were prepared (HM 340E, Microm, Wetzlar, Germany) and stained by the hematoxylin and eosin method (HE) using a multistainer (ST5020+CV5030, Leica, Wetzlar, Germany). The digitization was performed using a Pannoramic 250 Flash scanner (3DHistech, Budapest, Hungary). SlideViewer 2.6 software (3DHistech, Budapest, Hungary) was used for the morphometric analysis (the length of the villi, the crypt depth, the thickness of the mucosa, the thickness of the submucosa, and the thickness of the muscularis externa). The measurements were performed on three sections per animal, separated from each other by at least 1 cm, in 10 replicates per slide, and the mean values were statistically analyzed.

4.5. Immunofluorescence Staining

The segments for immunofluorescence testing were fixed in a 4% paraformaldehyde solution (pH 7.4). After one hour, the tissues were transferred to 0.1 M phosphate buffer (pH 7.4) for three days. The phosphate buffer was replaced every 24 h. The final step of tissue fixation involved their transfer to an 18% sucrose solution (pH 7.4) for 14 days at 4 °C. The intestinal segments were embedded in OCT Tissue-Tek (Sakura Finerek USA, Inc., Torrance, CA, USA) and, using a cryostat (CM 1860, Leica, Nussloch, Germany), cut at a temperature of −22 °C into 14 µm sections and attached to chrome-alum-coated slides. The staining steps are shown in Figure 8. To visualize the neurons in the ileum, protein gene product 9.5 (PGP 9.5) was used as a pan-neuronal marker. A list of the used antibodies is provided in Table 2. Microscopic analysis was performed using a Zeiss Axio Imager.M2 fluorescence microscope (Zeiss, Oberkochen, Germany), and a camera connected to a PC with ZEISS ZEN 3.0 Microscopy Software (Zeiss, Oberkochen, Germany) was used for image acquisition. To eliminate non-specific labeling, the preabsorption for the neuropeptide antisera with appropriate antigens was performed, and the omission and replacement tests were conducted. The testing performed noted no fluorescence. For the analysis, segments separated from each other by at least 200 µm were used to avoid counting the same cells.

Table 2. List of the antibodies used for immunofluorescence staining.

Primary Antibodies				
Antigen	**Species**	**Working Dilution**	**Code**	**Supplier**
CART	Rabbit	1:16,000	H-003-61	Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA
GAL	Guinea pig	1:2000	T-5036	Peninsula, San Carlos, CA, USA
nNOS	Rabbit	1:3000	PA1-033	ThermoFisher Scientific, Waltham, MA, USA
PGP 9.5	Mouse	1:1000	480012	
SP	Rat	1:150	8450-0505	BioRad, Hercules, CA, USA
VAChT	Rabbit	1:2000	H-V006	Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA
VIP		1:2000	ab22736	Abcam, Cambridge, United Kingdom
Secondary Antibodies				
Reagents		**Working Dilution**	**Code**	**Supplier**
Alexa Fluor 488	Donkey anti-mouse IgG (H + L)	1:1000	A21202	ThermoFisher Scientific, Waltham, MA, USA
Alexa Fluor 546	Donkey anti-rabbit IgG (H + L)		A10040	
	Goat anti- guinea pig IgG (H + L)		A11074	
	Goat anti-rat IgG (H + L)		A11081	

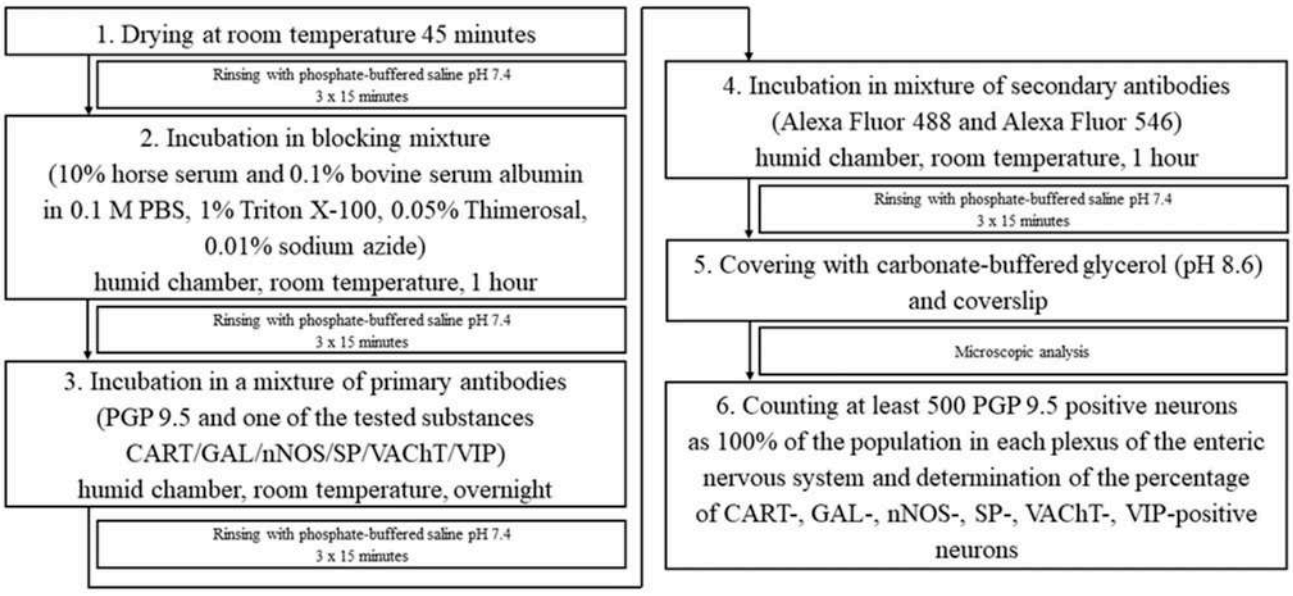


Figure 8. Immunofluorescence staining steps.

4.6. Statistical Analysis

Before statistical analysis, the assumption of linearity and normality was checked. For linearity testing, two-dimensional scatter plots of the analyzed variables were generated. The assumption of normality was confirmed using histograms and residual normality plots. The results are presented as mean ± SEM or mean ± SD. A one-factor analysis of variance (ANOVA) with the Schéffe post hoc test was used to demonstrate the absence or presence

of statistically significant differences. The differences were considered significant at a level of $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The study group was adopted as the independent variable, and the cytokine levels, morphometric indices, and the percentage of neurons immunoreactive toward the test substances were used as the dependent variables. The results were statistically analyzed using Statistica 13.3 software (TIBCO Software Inc., Palo Alto, CA, USA). GraphPad Prism 9.0.0 software (GraphPad Software, Boston, MA, USA) was used for visualization. The graphical abstract was created in BioRender, accessed on 14 October 2024 (BioRender.com/b68d975).

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