



Research Article

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Effect Of Multi-Walled Carbon Nanotubes on Structural and Cytochemical Parameters of Neurons in The Cerebral Ganglia of Gastropods *Limax Flavus*

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Abstract

Objective: The purpose of the study is evaluation of the effect of multi-walled carbon nanotubes on the structural and cytochemical parameters of neurons of cerebral ganglia of gastropods *Limax flavus* as a model of negative consequences of nanopollutants.

Material and Methods: Histological and histochemical study was conducted on 16 individuals of *Limax flavus*. The animals were intoxicated by applying 50 mg of multi-walled carbon nanotubes/individual to the body surface three times for 7 days.

Results: An increase in the ribonucleoprotein content of neuronal cytoplasm was observed in all three sections of the cerebral ganglion. This effect can be attributed to enhanced ribosomal activity, representing a compensatory response to cellular damage caused by the presence of nanoparticles in the animal's body. An increase in the activity of succinate dehydrogenase (an enzyme involved in both the tricarboxylic acid cycle and the respiratory electron transport chain) occurs in the mesocerebrum and metacerebrum. Regarding lactate dehydrogenase (an enzyme involved in the final stages of anaerobic glycolysis) significant differences between the control and experimental groups were observed only in the procerebrum. In the experimental group, the activity of glucose-6-phosphate dehydrogenase (an enzyme of the pentose phosphate pathway) and of reduced NADP dehydrogenase (an enzyme associated with extramitochondrial oxidation and nucleic acid synthesis) is elevated in all regions of the ganglion.

Conclusion: The ongoing violation leads to a change in neuromorphic homeostasis as a secondary phenomenon, which contributes to and aggravates the development of the primary action of nanopollutants in the body.

Keywords: Cerebral ganglia, Neurons, Gastropods, Multi-walled carbon nanotubes

Abbreviations: Dmax: Maximum Diameter of the Perikarya; Dmin: Minimum Diameter of the Perikarya; G-6-PDG: Glucose-6-Phosphate Dehydrogenase; IQR: Interquartile Range; LDH: Lactate Dehydrogenase; Me: Median; mesC: Mesocerebrum; metC: Metacerebrum; MWCNTs: Multi-Walled Carbon Nanotubes; NADPH dehydrogenase: Reduced Nicotinamide Adenine Dinucleotide Phosphate Dehydrogenase; P: Perimeter of the Perikarya; PC: Procerebrum; RNPs: Ribonucleoproteins; S: Cross-Sectional Area of the Perikarya; SDH: Succinate Dehydrogenase.

Introduction

In recent decades, the use of household chemicals and the chemicalization of food products have increased worldwide, affecting not only the functioning of individual organs and systems but also the human body as a whole [1-4]. Plastic nanoparticles and the abundance of products made from all types of plastic, including those used in food packaging, also contribute significantly [4-7]. All of this is driving researchers to seek new approaches to addressing the problems that arise.

One of the less studied systems in the body is the nervous system. Establishing cause-and-effect relationships at the level of morphological changes, particularly in the brain, under the influence of nanoparticles is a highly relevant and promising area, as the subtle mechanisms of such processes are not fully understood. Compared to vertebrates, the nervous system of invertebrates is more simply structured, but nevertheless, it is well adapted to changing environmental factors [8].

A number of animals, such as mollusks, annelids, and arthropods, are used as model objects for neurobiological research [9]. This can be explained by the common features of the organization of the nervous systems of invertebrates and vertebrates. Furthermore, common mechanisms of reception and methods of encoding information, as well as similar principles of the organization and integration of neuronal assemblies, are revealed. The relatively simple organization of the nervous system of invertebrates and the small number of nerve cells allow for the easy identification (mapping) of neurons responsible for the implementation of specific functions, which makes it possible to evaluate the influence of various regulatory factors on the functions of nerve centers independently of each other and thereby establish the cellular events underlying the coordination and interaction of their activity [10].

The nervous system of invertebrates is generally of the diffuse-nodular type, represented by large ganglia. Like vertebrates, it consists of two main parts: the central nervous system (a kind of "brain" with sensory and motor cells) and the peripheral nervous system, which includes the somatic nervous system, which regulates voluntary muscle movement, and the autonomic nervous system, responsible for cardiac contraction, intestinal peristalsis, vascular tone, and the function of certain glands [11].

Gastropods occupy a special place in neurobiology [9]. The relative ease of maintenance in laboratory conditions [12], the limited number of nerve cells (up to 100,000), and the similarity in the basic principles of the nervous system's structure and functioning to those of vertebrates [11] - all of this contributes to obtaining reliable information about the influence of various external experimental influences on the nervous system [13].

Slugs are the most highly developed gastropods, characterized by a nodal nervous system containing a small number of easily identifiable large neurons collected in individual ganglia - analogous

to the brain of vertebrates and humans - innervating various organ systems and providing communication between them [11].

The anteriormost ganglia - the paired cerebral ganglia - are considered to be analogous to the mammalian brain. They are the center for regulating the body's basic functions. They contain mixed nerves that innervate the main sensory structures of the head, including the lips, upper and lower tentacles, statocysts, and buccal mass, and are thus responsible for processing tactile and chemosensory signals [11]. In representatives of the Stylommatophora (stalked-eyed animals) *Helix* and *Limax*, the cerebral ganglia are more complexly structured than in other subclasses of gastropods. They are divided into three regions (divisions): the procerebrum (PC), the metacerebrum (metC), and the mesocerebrum (mesC) [14].

The absence in the literature of studies on the effect of microplastics on the nervous system of gastropods *Limax flavus* L., 1758 (slugs) was the reason for conducting this work using histological and histochemical methods in an adequate model.

The purpose of the study is evaluation of the effect of multi-walled carbon nanotubes on the structural and cytochemical parameters of neurons of cerebral ganglia of gastropods *Limax flavus* as a model of negative consequences of nanopollutants.

Material and Methods

Animals and Experimental Design

The study utilized material from 16 individuals of *Limax flavus*, belonging to the genus *Limax*, family *Limacidae*, order *Stylommatophora*, subclass *Pulmonata*, class *Gastropoda*, phylum *Mollusca*. The animals were collected in moist, ecologically clean areas of the Neman River floodplain in Grodno. To determine the species of the slugs, the identification guide by A. Sysoev and A. Schileyko "Land snails and slugs of Russia and adjacent countries" was used [15]. The collected animals were kept under laboratory conditions in moist plastic terrariums at room temperature on a bed of cabbage leaves. They were fed potato and carrot roots. Multi-walled carbon nanotubes (MWCNTs) (diameter 50-90 nm, length 1-2 μm , surface area 60-300 m^2/g , > 95%) (Guangzhou Hongwu Material Technology Co., Ltd., Jiangsu, People's Republic of China) (CAS No. 308068-56-6) were used in this study. Animals were intoxicated by applying 50 mg of MWCNTs per animal to their body surface three times daily for 7 days. All experiments were conducted in accordance with the regulations for work with experimental invertebrates. At the end of the experiment, 24 hours after the last application, in the morning (for time synchronization), the slugs were anesthetized with magnesium chloride [16] and cut into two halves. The anterior portion, containing the peripharyngeal ganglia ring, was used.

Histology and Histochemistry

For histological examination, specimens (5 experimental and 3 control) were fixed in Carnoy's solution. The samples were then

passed through increasing concentrations of alcohol, a mixture of alcohol and xylene, and pure xylene, and embedded in paraffin. Using a Leica RM2125 RTS microtome (Leica Microsystems GmbH, Germany), paraffin sections (5-10 μm thick) were prepared and stained using the Nissl method (with toluidine blue solution) to assess the size and shape of neurons in the cerebral ganglia of the studied mollusk species. After rinsing in distilled water, the preparations were passed through increasing concentrations of alcohol, carbol-xylene, and xylene, and embedded in polystyrene. To detect the content of ribonucleoproteins (RNP), paraffin sections were stained with gallocyanine-chrome alum using the Einarson method [17,18], followed by standard histological processing and embedding in polystyrene.

To study neuronal oxidative metabolism, samples of the anterior part of the mollusk body (5 experimental and 3 control), containing the peripharyngeal ring of cerebral ganglia, all three regions (procerebrum, mesocerebrum, and metacerebrum), were placed on strips of labeled filter paper, frozen, and stored in liquid nitrogen. Serial sections (10 μm thick) were obtained on a Leica CM1850 cryostat (Leica Microsystems GmbH, Germany) at -18°C . To differentiate ganglia, the preparations were pre-stained with methylene blue. The sections were mounted on glass slides and dried for 1 hour at room temperature. Next, the cryostat sections were processed to detect the activity of oxidoreductases associated with the Krebs cycle: succinate dehydrogenase (SDH, EC 1.3.5.1) [19], with glycolysis - lactate dehydrogenase (LDH, EC 1.1.1.28) [20], with extramitochondrial oxidation and synthesis of nucleic acids - reduced nicotinamide adenine dinucleotide phosphate dehydrogenase (NADPH dehydrogenase, EC, 1.6.5.10) [21], with the pentose phosphate pathway - glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) [22]. After staining to detect dehydrogenase activity, sections were washed in distilled water, fixed for 30 min in 10% neutral formalin, dehydrated in increasing concentrations of alcohol, cleared in xylene, and embedded in polystyrene [17,21].

Microphotography and Morphometric Analysis of Neurons

The study of histological and histochemical preparations, their description, microphotography and morphometry were carried out at different magnifications of an Axioskop 2 plus microscope (Zeiss, Germany), a built-in digital video camera Leica DFC320 (Leica Microsystems GmbH, Germany) and the Image Warp computer image analysis program (Bit Flow, USA).

A quantitative assessment of the size and shape of neurons was performed by tracing the contours of their perikarya on the monitor screen with a cursor, obtaining the following parameters:

minimum and maximum diameters, perimeter, area, volume of neurons ($4/3\pi\sqrt{(S/\pi)^3}$, where S is the cross-sectional area of the perikarya), form factor ($4\pi S/P^2$, where S is the cross-sectional area of the perikarya, P is the perimeter of the perikarya) and elongation factor ($D_{\text{max}}/D_{\text{min}}$, where D_{max} is the maximum diameter of the perikarya, D_{min} is the minimum diameter of the perikarya).

Cytophotometric analysis of histochemical preparations was performed by determining the optical density of the resulting chromogen precipitate in the cytoplasm of the neurons studied, at the absorption maximum of the stained reaction products. Relative activity and substance content were expressed in units of optical density.

Statistics

The primarily data was obtained by nonparametric statistics using the Statistica 10.0 program (Stat Soft, Inc., USA). Quantitative results were presented as "Me $\pm$ IQR", where Me is the median, IQR is the interquartile range. Comparison of groups on one basis was carried out using the Mann-Whitney U-test for independent samples. Differences between groups were considered statistically significant if the probability of an erroneous estimate did not exceed 5% ($p < 0.05$, where p is the critical value of the significance level) [23].

Results and Their Discussion

The results of histological and histochemical studies showed that when nanoparticles are applied to the animal's body, certain changes occur in the neurons of the cerebral ganglion of the mollusk *Limax flavus*.

Morphometric studies revealed virtually no statistically significant differences in the perikarya and nuclei of nerve cells in all three ganglion regions (procerebrum, mesocerebrum, and metacerebrum) among the experimental animals. This may indicate the absence of profound and severe neuronal changes following exposure to nanoparticles.

A completely different picture was revealed by the histochemical study. In the experiment, the amount of ribonucleoproteins in the cytoplasm of procerebrum neurons increased by 77.1% ($p = 0.02$) compared to the control group (Figures 1, 2). Nanoparticles have an effect on the mesocerebrum, since the RNPs content in the cytoplasm of neurons in this region increases by 80% ($p = 0.008$) (Figures 1, 2). A similar picture is revealed in the metacerebrum, where the amount of ribonucleoproteins in the cytoplasm of neurons in the experimental group increases by 40% ($p = 0.002$) (Figure 2).

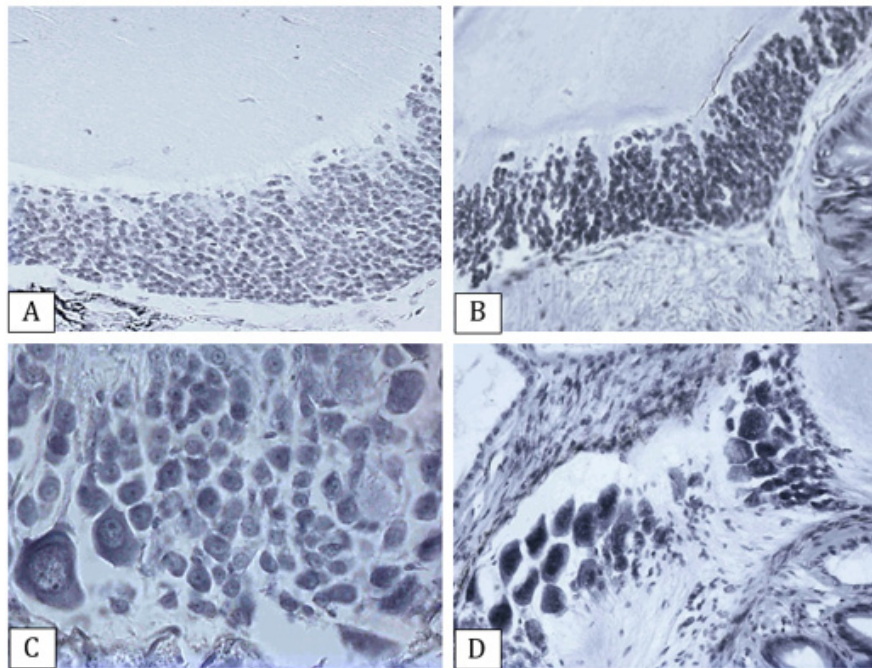


Figure 1: RNPs content in the cytoplasm of neurons of the procerebrum (A, B) and mesocerebrum (C, D) of the cerebral ganglia of the slug *Limax flavus* in the control (A, C) and experimental (B, D) groups. Stained according to Einarson. Digital micrograph. Magnification: $\times 200$ (A, B), $\times 400$ (A, B).

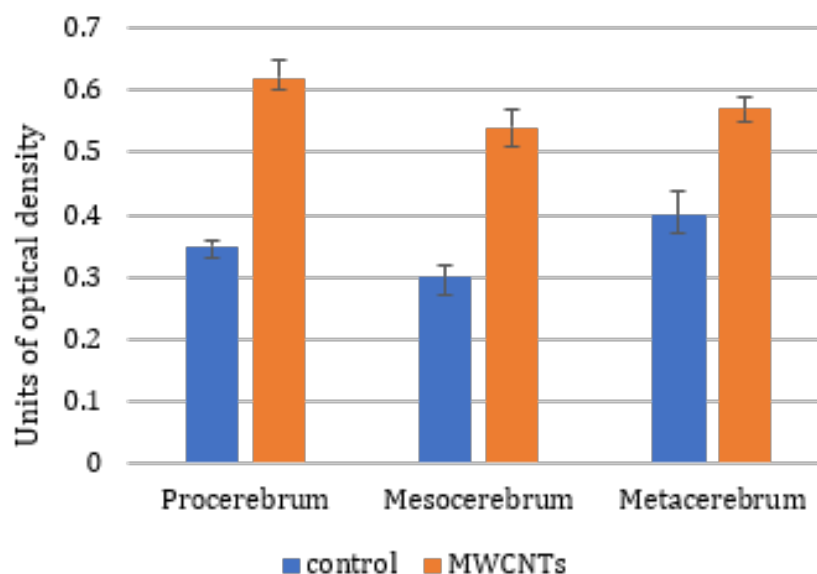


Figure 2: Changes in the RNPs content in the cytoplasm of neurons in different regions of the cerebral ganglia of *Limax flavus* slugs exposed to multiwalled carbon nanotubes. The horizontal axis represents three regions of the cerebral ganglia, and the vertical axis represents the optical density of the chromogen obtained in the corresponding histochemical reaction. Data are presented as Me $\pm$ IQR; * - $p < 0.05$ compared to the control group (Mann-Whitney U-test).

Thus, statistically significant differences in RNPs content in the neuronal cytoplasm were detected in all three regions of the cerebral ganglion, with increases ranging from 40% to 80%. This phenomenon may be associated with increased ribosome activity

as a compensatory response to cellular damage caused by the presence of nanoparticles in the body. An increase in RNPs content may indicate increased activity of the granular endoplasmic reticulum and ribosomes, reflecting increased activity of the entire

cellular protein-synthesizing apparatus. This, however, does not lead to energy deficiency or, ultimately, morphological damage to the neurons themselves [24].

When studying the oxidative metabolism of procerebrum neurons in cryostat sections in mollusks, it was found that in the experiment there is an increase in the activity of LDH by 20% ($p = 0.002$), G6PDH by 8.0% ($p = 0.005$) and NADPH dehydrogenase by 4.9% ($p = 0.04$). Enzymatic activity in the perikarya of

mesocerebrum neurons of the cerebral ganglion of the mollusc is as follows: there is an increase in the activity of SDH by 15.8% ($p = 0.02$), G6PDH by 7.7% ($p = 0.005$) and NADPH dehydrogenase by 6.1% ($p = 0.04$) in the experimental animals. The activity of enzymes in the perikarya of neurons of the metacerebrum of the cerebral ganglion of the mollusc in the experiment: there is an increase in the activity of SDH by 11.1% ($p = 0.02$) (Figure 3), G6PDH by 8.3% ($p = 0.004$) and NADPH dehydrogenase by 11.1% ($p = 0.005$) (Figures 3, 4).

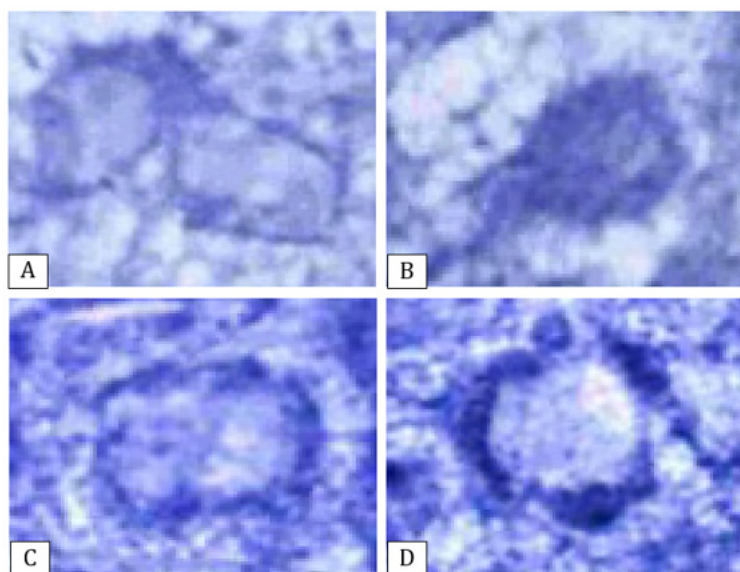
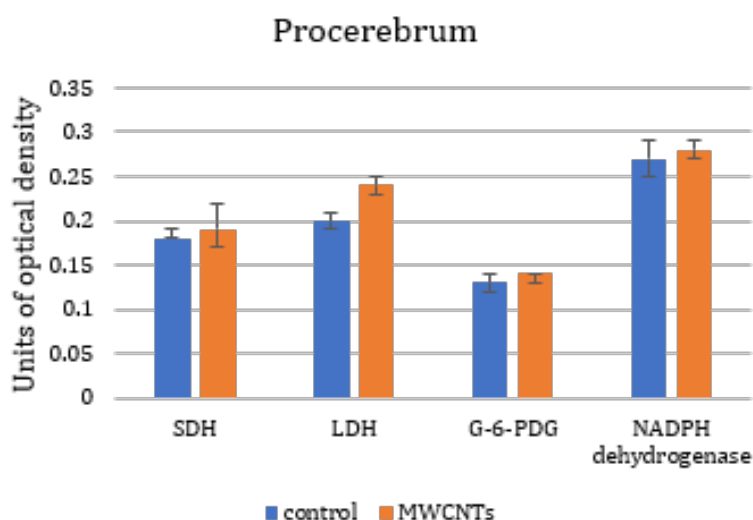


Figure 3: Activity and localization of SDH (A, B) and NADPH dehydrogenase in the cytoplasm of neurons of the metacerebrum of cerebral ganglia of the slug *Limax flavus* in the control (A, C) and experimental (B, D) groups. Staining according to Nachlas et al. (A, B), staining according to Nachlas, Walker and Seligman (C, D). Digital micrograph. Magnification: $\times 800$.



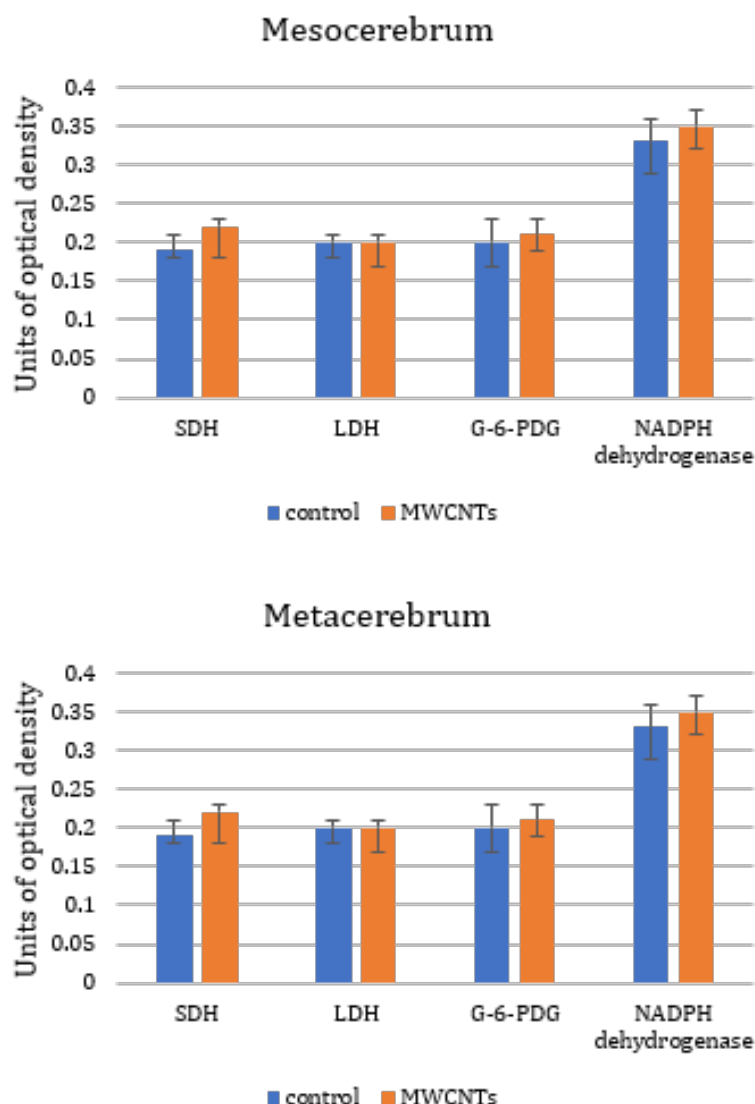


Figure 4: Changes in cytoplasmic enzymatic activity of neurons in different regions of the cerebral ganglia of *Limax flavus* slugs exposed to multiwalled carbon nanotubes. The studied enzymes are shown on the horizontal axis, and the optical density of the chromogen obtained in the corresponding histochemical reaction is shown on the vertical axis. Data are presented as Me $\pm$ IQR; * - $p < 0.05$, ** - $p < 0.01$ compared to the control group (Mann-Whitney U-test).

Thus, the study results showed that microplastics influence the activity of the enzymes studied. There is an increase in the activity of SDH, an enzyme associated with the tricarboxylic acid cycle [19], in the mesocerebrum and metacerebrum. This can be explained by the fact that the mesocerebrum is responsible for controlling spermatogenesis and mating behavior in males, while the metacerebrum is responsible for tentacle retraction and modulation of feeding behavior [25,26].

For LDH, an enzyme involved in the final stages of glycolysis occurring under anaerobic conditions [20], there are significant differences between the control and the experimental conditions only in the procerebrum region. It is likely that changes in enzyme

activity, particularly mitochondrial ones, are not critical enough to trigger anaerobic glycolysis, since this region acts as an olfactory information processing center [26].

The activity of G6PDH, an enzyme of the pentose phosphate pathway [22], was increased in the experiment in all parts of the ganglion. There was a significantly different increase in the activity of NADPH dehydrogenase, an enzyme that catalyzes extramitochondrial redox reactions and is coupled with NO synthase [21], among all the ganglion parts studied.

Thus, the greatest toxic effect of microplastics, according to histochemical data, was noted in the procerebrum and metacerebrum, and the least in the mesocerebrum.

The toxic effects of nanoparticles depend on experimental conditions, such as exposure time, concentrations, cellular and tissue-specific responses, animal species, and nanoparticle types [27], specifically on the nature and structure of the nanoparticles [28]. It is currently impossible to definitively determine the precise mechanisms underlying these effects in this model. However, it can be stated with certainty that the resulting disruption leads to altered neuromorphic homeostasis as a secondary phenomenon, which facilitates and exacerbates the primary effects of nanopollutants in the body.

Conclusion

MWCNTs act as a stress factor that, without damaging neuronal structure, forces the nervous system to switch to a more intense metabolic mode. This demonstrates the high sensitivity of invertebrate neurons to nanocontaminants and confirms the suitability of *Limax flavus* as an adequate model for assessing nanoparticle neurotoxicity.

Author Contributions

All authors made the same contribution to this study and the preparation of the article: to conceptualization (ideas, formulation, or development of research goals and objectives), analysis, writing original draft (preparation, creation, and/or presentation of the published work, specifically writing the initial draft), review and editing (preparation, creation, and/or presentation of the published work by the research group, specifically critical review, commentary, or revisions), and visualization (preparation, creation, and/or presentation of the published work, specifically data visualization/presentation).

Conformity with the Principles of Ethics

The study was performed in accordance with the principles of bioethics.

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None.

Conflict of Interest

Authors declare that they have no financial or personal conflicts of interest that could inappropriately influence the conduct of this research.

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