Carbon-based nanomaterials cause toxicity by oxidative stress to the liver and brain in Sprague-Dawley rats

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Abstract

Carbon-based nanomaterials have important research significance in various disciplines, such as composite materials, nanoelectronic devices, biosensors, biological imaging, and drug delivery. Recently, the human and ecological risks associated with carbon-based nanomaterials have received increasing attention. However, the biosafety of carbon-based nanomaterials has not been investigated extensively. In this study, we used different types of carbon materials, namely, graphene oxide (GO), single-walled carbon nanotubes (SWCNTs), and multi-walled carbon nanotubes (MWCNTs), as models to observe their distribution and oxidative damage in vivo. The results of histopathological and ultrastructural examinations indicated that the liver and lungs were the main accumulation targets of these nanomaterials. SR- -XRF analysis revealed that SWCNTs and MWCNTs might be present in the brain. This shows that the three types of carbon-based nanomaterials could cross the gas–blood barrier and eventually reach the liver tissue. In addition, SWCNTs and MWCNTs could cross the blood–brain barrier and accumulate in the cerebral cortex. The increase in ROS and MDA levels and the decrease in GSH, SOD, and CAT levels indicated that the three types of nanomaterials might cause oxidative stress in the liver. This suggests that direct instillation of these carbon-based nanomaterials into rats could induce ROS generation. In addition, iron (Fe) contaminants in these nanomaterials were a definite source of free radicals. However, these nanomaterials did not cause obvious damage to the rat brain tissue. The deposition of selenoprotein in the rat brain was found to be related to oxidative stress and Fe deficiency. This information may support the development of secure and reasonable applications of the studied carbon-based nanomaterials.

Full Text

Preamble

Carbon-based nanomaterials cause toxicity by oxidative stress to the liver and brain in Sprague-Dawley rats

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Carbon-based nanomaterials hold significant research importance across various disciplines, including composite materials, nanoelectronic devices, biosensors, biological imaging, and drug delivery. Recently, the human and ecological risks associated with these materials have received increasing attention, yet their biosafety has not been investigated extensively. In this study, we used different types of carbon materials—graphene oxide (GO), single-walled carbon nanotubes (SWCNTs), and multiwalled carbon nanotubes (MWCNTs)—as model systems to observe their distribution and oxidative damage in vivo.

Histopathological and ultrastructural examinations indicated that the liver and lungs were the primary accumulation sites for these nanomaterials. SR-μ-XRF analysis revealed that SWCNTs and MWCNTs might also be present in the brain, demonstrating that all three types of carbon-based nanomaterials could cross the gas-blood barrier and reach liver tissue. Additionally, SWCNTs and MWCNTs could cross the blood-brain barrier and accumulate in the cerebral cortex. The observed increase in ROS and MDA levels, coupled with decreased GSH, SOD, and CAT levels, suggested that these nanomaterials induce oxidative stress in the liver, indicating that direct instillation into rats could trigger ROS generation. Iron (Fe) contaminants in the nanomaterials represented a definite source of free radicals. However, the nanomaterials did not cause obvious damage to rat brain tissue. Notably, selenoprotein deposition in the rat brain was found to be related to oxidative stress and Fe deficiency. These findings may support the development of secure and reasonable applications for the studied carbon-based nanomaterials.

Keywords: carbon-based nanomaterials; oxidative stress; trace element distribution; TEM; SR-μ-XRF

Introduction

Nanotechnology has revolutionized fields ranging from manufacturing to healthcare, with carbon-based nanomaterials emerging as a global research hotspot [1-3]. Ideal carbon nanotubes (CNTs) are novel nanomaterials that typically adopt

a multilayer composite structure ranging from a single layer to over 100 layers. CNTs with one graphene layer are called single-walled carbon nanotubes (SWCNTs), while those with multiple layers are called multiwalled carbon nanotubes (MWCNTs). Owing to their excellent aspect ratios and electrical, mechanical, and physicochemical properties, they have been applied in composite materials [4,5], nanoelectronic devices [6], biological sensors [7], biological imaging [8,9], and biomedicine [10,11]. Carbon-based nanomaterials can also serve as novel transport vehicles for various peptides, proteins, plasmid DNAs, and siRNA in the biomedical field [12,13]. CNTs demonstrate good modifiability, showing promising applications in diagnostics, targeting, and drug loading. It is worth mentioning that worldwide production of MWCNTs and SWCNTs was approximately 2,000 tons and 6 tons, respectively, in 2013 [14], and is expected to exceed 20,000 tons combined in 2022.

The increasing popularity and production of CNTs will inevitably lead to environmental and human exposure, making the toxicity of carbon-based nanoparticles a notable challenge requiring further investigation. Although the toxicological mechanism of carbon-based nanomaterials remains unclear, oxidative stress is considered one of the main causes of their toxic effects [15–17], characterized by excess reactive oxygen species (ROS) resulting from redox state imbalance [18]. Previous studies have demonstrated that carbon-based nanomaterials cause significant damage to the liver, kidneys, and brain—organs sensitive to oxidative stress [19–22]. Additionally, instillation of carbon-based nanoparticles in rats can result in lung injury with obvious inflammatory and fibrotic features, potentially due to the accumulation of black foreign substances in the lungs. Wang et al. found that SWCNTs accumulated in small doses in vascular endothelial cells and in bone, stomach, and kidney tissues for extended periods. Furthermore, MWCNTs have been reported to cross most biological barriers after tail vein administration, including the blood–brain barrier (BBB) [24,25]. However, due to various exposure routes, there is no significant difference in the distribution of carbon-based nanomaterials in the body [23], which is closely related to their transport capacity [26].

Several neurotoxicological mechanisms have been associated with cell homeostasis, trace elements [27–30], and free radical production [31]. The interaction between nanomaterials and trace elements, particularly selenium (Se)—known as an antioxidant—has an antagonistic effect on nanomaterials [32]. Synchrotron micro-X-ray fluorescence (μ-XRF) with high spatial resolution is an excellent technique for mapping elemental distributions [33,34]. However, the mechanism underlying the spatial distribution and cross-cellular transport of carbon-based nanomaterials in vivo remains unclear, especially regarding cytotoxicity caused by oxidative stress.

In light of these considerations, this study used Sprague–Dawley (SD) rats intratracheally instilled with different concentrations of graphene oxide (GO), SWCNTs, and MWCNTs as models to confirm the toxicity of carbon-based nanomaterials, including oxidative stress and distribution patterns. Their location

in tissues was determined through histopathological, ultrastructural, and SR-μ-XRF examinations. We then focused on oxidative stress and antioxidant status in the liver and brain, and evaluated the spatial distribution of major trace elements calcium (Ca), iron (Fe), and selenium (Se) in the rat brain. These data support the development of secure and reasonable applications.

II. Materials and Methods

A. Properties of Carbon-Based Nanomaterials

GO was prepared using the improved Hummers method [35,36], while SWCNTs and MWCNTs were synthesized using catalytic chemical vapor deposition technology [37]. All carbon-based nanomaterials were obtained from the Institute of Chemistry, Chinese Academy of Sciences. The characterization results are summarized in Table 1 . After UV irradiation for 2 h, the carbon-based nanomaterials were suspended in normal saline to obtain concentrations of 50, 100, and 200 μg/mL. The suspension was ultrasonicated for 20 min, with a break every 10 min before use. The morphology and structure of the samples were observed using transmission electron microscopy (TEM, Hitachi-7650).

Table 1. Characterization of Carbon-Based Nanomaterials

Samples and characterization
GO: diameter <500 nm
SWCNTs: purity >90 wt.% carbon content and <10 wt.% Fe catalyst, diameter: 2 nm, length: 5–15 μm
MWCNTs: purity >95 wt.% carbon content and <5 wt.% Fe catalyst, diameter: 10–20 nm, length: 5–15 μm

B. Animals and Treatment

Sixty normal male SD rats were purchased from the Experimental Animal Research Center of the Chinese Academy of Sciences (Shanghai, China) and housed in cages under a 12 h light/dark cycle. The environmental conditions were maintained at 23–26°C with 50–60% relative humidity. Pure water and sterilized food were provided ad libitum.

The SD rats were randomly divided into 10 groups (n = 6 each):
(1) Normal group (GI): received normal saline
(2) GO-treated group (GII): received GO (50, 100, and 200 μg/mL)
(3) SWCNT-treated group (GIII): received SWCNTs (50, 100, and 200 μg/mL)
(4) MWCNT-treated group (GIV): received MWCNTs (50, 100, and 200 μg/mL)

A 1.0 mL suspension was instilled into the trachea of anesthetized rats using chloral hydrate. The rats recovered to normal health within 30 days and were then euthanized. The lungs, liver, and brain were immediately collected and processed according to analytical requirements. All procedures were performed

in accordance with the standards of the China-Japan Friendship Hospital and followed strict principles for pain relief in rats.

C. Pathological and EM Examination

Lung and liver tissues were removed, washed with aqueous phosphoric acid solution, and fixed with 10% neutral buffered formalin (Shanghai Guoyao Chemical Company, China) for 2 days. After phosphate-buffered saline (PBS) washing, tissues were dehydrated in graded alcohol solutions, paraffin-embedded, sectioned at 5 µm thickness, and stained with hematoxylin–eosin (H&E). Pathological changes in lungs and liver were observed under a microscope (Leica Microsystems, Germany).

D. Transmission Electron Microscopy (TEM)

Liver blocks were fixed with 2.5% glutaraldehyde (Shanghai National Pharmaceutical and Chemical Company) for 4 h, washed with PBS three times, and treated with 1% cerium oxide for 1 h. Following standard protocols, samples were washed with PBS, dehydrated in graded alcohol series, and embedded in epoxy resin. Ultrathin sections were cut using an ultramicrotome (UC6, Leica), mounted on copper grids, stained with uranyl acetate and lead citrate, and observed using TEM (Hitachi-7650).

E. Evaluation of Oxidative Stress and Antioxidant Status in Rat Liver and Brain Tissues

Liver and brain tissues were homogenized in 50 mM Tris-10 mM EDTA buffer (pH 7.4) and centrifuged at 13,000×g at 4°C for 30 min. Indices of oxidative stress and antioxidant status—including superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), glutathione (GSH), lactate dehydrogenase (LDH), and ROS—were measured. SOD and CAT activities were measured using assay kits from Nanjing Jiancheng Bioengineering Institute and Beyotime Institute of Biotechnology, respectively. Lipid peroxidation products were analyzed using an MDA assay kit (Nanjing Jiancheng Bioengineering Institute). GSH levels were measured according to the manufacturer's protocols in the GSH assay kit (Nanjing Jiancheng Bioengineering Institute). LDH leakage, used to assess membrane damage, was determined using a commercial LDH kit (Beyotime Institute of Biotechnology, Jiangsu). ROS levels were determined using ROS reagent kits (Beyotime Institute of Biotechnology, China). All procedures were performed according to manufacturer instructions, with a minimum of three trials per measurement.

F. Synchrotron Radiation-Induced Micro X-Ray Fluorescence Analysis (SR µ-XRF)

The distributions of Ca, Fe, and Se in brain sections were studied using µ-XRF at BL15U1 at the Shanghai Synchrotron Radiation Facility (SSRF) [27,38]. In

the storage ring, the electron beam energy was approximately 3.5 GeV with a beam current of approximately 200 mA. During experiments, sample slices were placed on a holder and moved in both X- and Y-directions by a stepper motor driven by a microcomputer. Elemental distributions were continuously observed at 30 µm steps in both directions. Ten parallel scans were performed on brain samples at a resolution of 30 µm × 30 µm from edge to center, covering an area of approximately 5.5 × 8.0 mm^2. The emission energy was 16.5 keV with a collection speed of 1.0 s/step. Elemental fluorescence intensities were normalized to ionization chamber signals collected simultaneously. X-ray spectra were analyzed using IGORPRO6.10 (Waveguide Materials Co., Ltd.).

G. Statistical Analysis

All data are presented as mean ± standard deviation. One-way ANOVA and Dunnett' s test were used for group comparisons. A significant difference was defined as $P < 0.05$.

III. Results

A. Carbon-Based Nanomaterial Characterization

The morphology and structure of carbon-based nanocrystals were determined using TEM (Fig. 1 [Figure 1: see original paper]). GO particle size was approximately 400 nm with a tendency to form clusters. SWCNT and MWCNT samples were easily entangled and difficult to separate even with ultrasonic vibration (Figs. 1B and 1C), consistent with previous reports [39,40].

B. Pulmonary Inflammatory Cell Infiltration

Alveolar volume was normal with no obvious pulmonary artery dilation after normal saline treatment (Fig. 2A [Figure 2: see original paper]). Compared with the normal group (GI), more black foreign bodies appeared in the lungs of rats treated with carbon-based nanomaterials (GII, GIII, and GIV), accompanied by substantial inflammatory cell infiltration (Figs. 2B–2D). A few black foreign bodies were observed in the alveolar ducts of the GII group, characterized by irregular sizes and alveolar collapse. Significant black foreign bodies remained visible in the bronchi of GIII and GIV groups, with neutrophils and alveolar wall thickening present in the alveolar cavity. These results demonstrate that carbon-based nanomaterials can induce inflammatory lung injury.

C. Hepatic Inflammatory Cell Infiltration

Histopathological images revealed abnormal liver tissue structure after carbon-based nanomaterial exposure (Figs. 3A–3D [Figure 3: see original paper]). Compared with GI, irregular liver tissue arrangements, cytoplasmic vacuole changes, and portal vein congestion and exudation were observed after SWCNT and

MWCNT exposure, accompanied by inflammatory cell exudation and local calcification. No significant changes were observed in the GII group. TEM examination (Figs. 4A–4D [Figure 4: see original paper]) revealed pathological changes including nuclear pyknosis, mitochondrial swelling, and loss of mitochondrial cristae. Numerous small black substances were observed in mitochondrial cavities of GII rats, while large SWCNT clusters appeared in GIII mitochondria, with considerable black material also present in GIV rat mitochondria. These results indicate that carbon-based nanoparticles with different morphologies can be transported from lungs to liver tissue via blood circulation.

To confirm inflammatory damage suggested by histopathology, oxidative stress levels were measured in liver tissues (Figs. 5A–5F [Figure 5: see original paper]). ROS levels increased significantly after carbon-based nanomaterial treatment in a concentration-dependent manner. MDA levels were elevated in GII, GIII, and GIV compared with GI, indicating lipid peroxidation. SOD and CAT activities, key antioxidant enzymes, decreased after exposure. GSH levels showed a reduction tendency similar to SOD and CAT. Additionally, LDH generation decreased in treated groups compared with GI. These findings demonstrate that carbon-based nanomaterials cause inflammatory damage and oxidative stress in rat liver tissues. Notably, our samples contained large amounts of Fe ions introduced during synthesis that were difficult to remove completely.

D. Neural Inflammatory Cell Infiltration

To investigate whether carbon-based nanomaterials cause brain damage via the blood system, oxidative stress responses and trace element spatial distribution were assessed. Oxidative pressure responses in rat brain after exposure are shown in Figs. 6A–6F [Figure 6: see original paper]. MDA and ROS levels were slightly increased, while LDH, SOD, GSH, and CAT levels decreased. Different concentrations of carbon-based nanomaterials exerted some oxidative damage effects, though to a lesser degree than in liver tissue.

The spatial distributions of Ca, Fe, and Se in rat brain after treatment were detected using SR μ-XRF (Figs. 7–9 [Figure 7: see original paper], [Figure 8: see original paper], [Figure 9: see original paper]). Elemental images corresponded well with brain section morphology. Ca distribution was similar across all groups, being most abundant in the cortex and moderately present in other regions, with no calcification observed after nanomaterial exposure. Fe was most abundant in the cortex of control brains (Fig. 8A) but decreased significantly after nanomaterial treatment (Figs. 8B–8D). High-concentration Fe spots observed in SWCNT- and MWCNT-exposed groups likely originated from Fe catalyst content (<10 wt.% in SWCNTs, <5 wt.% in MWCNTs). Interestingly, Se content increased significantly in treated brain sections compared with GI, with granular Se precipitation observed after carbon-based nanomaterial treatment (Figs. 9B–9D). These results suggest that oxidative stress in rat brain after treatment might be caused by trace element Fe entry and selenoprotein precipitation.

IV. Discussion

Carbon-based nanoparticles have important research value in integrated tumor diagnosis and treatment systems due to their unique physical and chemical properties [41,42]. However, they have shown toxicological effects on human health and the environment [43,44], and their safety in medical applications remains controversial [45,46]. Prior studies demonstrated that high concentrations of SWCNTs and MWCNTs cause inflammatory responses and lung tissue injury [47,48]. Therefore, with nanosafety considerations in mind, we used three different carbon-based nanomaterials—GO, SWCNTs, and MWCNTs—as research objects in an in vivo animal experiment to confirm enrichment and oxidative damage in rat lung, liver, and brain tissues.

Studies have identified the liver, kidney, and lungs as important targets for CNT accumulation, with metabolites excreted through urine [49,50]. Wang et al. [23] found SWCNTs present in vascular endothelial cells and in bone, stomach, and kidney tissues. Warheit [51] and Lam [52] also studied lung injury induced by respiratory tract injection of unpurified SWCNTs. In this study, histopathological and TEM results clearly observed carbon-based nanomaterials in lungs and liver, showing enrichment in lungs with inflammatory response after pulmonary instillation. Small amounts of black foreign bodies in rat liver indicated that nanomaterials in different forms can cross the gas–blood barrier to reach liver tissue (Fig. 10 [Figure 10: see original paper]), consistent with previous results [25,53].

Compared with liver tissue, brain tissue has a tight BBB that prevents various soluble particulates from entering [54,55]. SR-μ-XRF results indicated that SWCNTs and MWCNTs may cross the BBB and accumulate in the rat brain cortex, meaning they can enter systemic circulation—consistent with previous findings [24,56]. Wang et al. [24] reported that MWCNTs broke through most biological barriers, including the BBB, after caudal vein administration. CNT distribution in the body differs significantly among exposure routes [23], with transport likely depending on blood flow and macrophage activity [26]. Inflammatory cell infiltration, nuclear pyknosis, mitochondrial swelling, and mitochondrial cristae loss observed in rat liver tissues were related to oxidative stress.

Oxidative stress is a key factor mediating inflammatory cell responses [57]. ROS, as by-products of energy consumption, play crucial roles in maintaining homeostasis [58], but these steady states are easily disrupted by external environmental stresses. In our study, ROS levels increased in both liver and brain, indicating that direct instillation of carbon-based nanomaterials could induce ROS generation. The SWCNT and MWCNT samples contained less than 10 wt.% and 5 wt.% Fe catalyst, respectively. Transition elements serve as main sources of free radicals and have significant toxicological effects [59]. High ROS levels can cause serious damage to lipids, proteins, DNA, and other tissues, resulting in cardiovascular and neurological diseases [15]. Our results showed that carbon-based nanomaterials reduced LDH levels and increased MDA levels in

liver and brain, damaging cell membrane biological function through oxidative injury. GSH, a ubiquitous sulfhydryl-containing molecule in cells, is affected by ROS [60], and decreased GSH levels in liver after nanomaterial exposure may indicate cellular damage. The extra ROS likely induced substantial inflammatory cell infiltration in rat liver tissue, increased mitochondrial volume, and caused mitochondrial cristae loss. ROS can be eliminated by scavenging enzymes such as SOD and CAT, which convert superoxide anions to hydrogen peroxide and water [61,62]. However, SOD and CAT activities decreased markedly in the presence of carbon-based nanoparticles, though antioxidants could still scavenge excess ROS to maintain balance.

In contrast, some researchers have discussed the safety of these materials and insisted CNTs are harmless to humans [63–65]. In our study, MDA and ROS levels increased only slightly in rat brain after exposure, with GSH, CAT, and SOD activities decreasing slightly—changes closely related to oxidative stress. Se is well known for its antioxidative capacity, with most selenoprotein found in the brain distributed mainly in the olfactory bulb, cortex, hippocampus, and cerebellum [32]. Ingold et al. [66] reported that selenocysteine-containing GPX4 protects neurons from oxidative stress and ferroptosis. Our study found that selenoprotein deposition in rat brain is related to oxidative stress and Fe deficiency.

V. Conclusion

Carbon-based nanomaterials play critical roles in composite nanoelectronic devices, biosensors, biological imaging, and drug delivery. While they pose certain human and ecological risks that make their medical applications controversial, this study used GO, SWCNTs, and MWCNTs as models to observe their in vivo distribution and oxidative damage. Histopathological and ultrastructural studies indicated that the liver and lungs were primary accumulation sites. SR-μ-XRF analysis showed that SWCNTs and MWCNTs may also reach the brain, demonstrating that these nanoparticles can cross the gas–blood barrier to reach liver tissue and cross the BBB to accumulate in the cerebral cortex. The three nanomaterial types caused liver oxidative stress, evidenced by increased ROS and MDA levels and decreased GSH, SOD, and CAT levels, suggesting that direct instillation induces ROS generation. Fe contaminants in these materials represent a source of free radicals. However, carbon-based nanoparticles did not cause discernible brain tissue damage in rats. Selenoprotein deposition in rat brain was shown to be related to oxidative stress and Fe deficiency.

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Data Availability: All data are included in the manuscript.

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

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Note: Figure translations are in progress. See original paper for figures.