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RESEARCH ARTICLE

Oxidative damage in the kidney and brain of mice induced by different nano-materials

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Abstract **With the rapid development of nanotechnology, nanomaterials have been used in numerous fields. However, these nanomaterials could also result in risk for human and environmental health. To make a comparison of the health effects of three different kinds of nanomaterials, 28 male BALB/c mice were randomly divided into four groups. Three experimental groups were exposed to different kinds of nanomaterials including graphene, graphene oxide and single wall carbon nanotubes (SWCNTs) by intraperitoneal injection while the control group received a saline injection. The exposure dose of experimental groups was 4mg/kg. After seven days, sections of mice kidney were taken, the organ coefficient of both kidney and brain was counted, and the reactive oxygen species (ROS) level, glutathione (GSH) and malondialdehyde (MDA) content was measured. Our results showed that in the experimental groups, the organ coefficient and GSH content in mice kidneys and brains decreased, whereas the ROS level and MDA content increased, when compared with the control. The graphene oxide group was statistically significant ($p < 0.05$), while the SWCNTs group had extremely significant difference ($p < 0.01$). Morphological changes in the kidney were also seen in the experimental groups. These results demonstrate that oxidative damage to mice kidneys and brains induced by SWCNTs and graphene oxide is more severe than graphene. The degree of damage caused by these three typical nanomaterials is different, probably due to several parameters including particle size, surface character, and shape.**

Keywords graphene, graphene oxide, single wall carbon nanotubes, oxidative damage

Introduction

Rapid advances in nanotechnology saw numerous engineered nanomaterials being fabricated. Since the start of this century, with the rapid development of nanotechnology, nanomaterials have been widely used in many fields including health care products, electronics, photonics, biomedicine, cosmetics, garments, sensors, catalysts, etc. Accompanying the benefits of nanomaterials, there could be a concomitant increase in the risk for human and environmental health, as a result of its small size effect, surface effect, quantum size effect and macroscopic quantum tunneling effect.

Graphene is a single-atom-thick sheet of sp^2-bonded carbon atoms in a closely packed honeycomb two-dimensional lattice structure (Liu et al., 2012), the thickness is only 0.35 nm and is the thinnest two-dimensional material in the world. It is considered to be the most optimal nanomaterial with the advantages of high- performance, low-cost and good machinability. The study showed that after 24 h of exposure to graphene (G), the metabolic activity of Pheochromocytoma cells decreased rapidly. Moreover, reactive oxygen species (ROS) were also generated in a concentration and time-dependent manner after exposure to G (Zhang et al., 2010); Graphene oxide (GO) is an important derivative of graphene whose surface has a lot of oxygenic functional groups (epoxy group, hydroxyl, carboxyl), it has good hydrophilicity, and greatly extended its application in the field of material, chemistry and sensor (Zhang and Cui, 2012). Sangiliyandi Gurunathan found that when cells were exposed to GO, they produced superoxide radical anions and lost their viability, and that GO demonstrated dose-dependent antibacterial activity against *Pseudomonas aeruginosa* cells through the generation of reactive oxygen species (Gurunathan et al., 2012). Single wall carbon nanotubes (SWCNTs) have a single-walled tube structure with good symmetry and unicity,

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and its chief applications are in medical science, for hydrogen storage and electronic material, etc. (Wang and Jiang, 2008). As early as 2004, Lam had reported that when mice were treated with 0, 0.1 and 0.5 mg SWCNTs and euthanized 7 days or 90 days after the treatment, all the nanotubes could induce epithelial cell granuloma and react in a dose-dependent manner, the lesions persisted and were more pronounced in the 90-d groups (Lam et al., 2004). Also the effects of SWCNTs on pulmonary oxidative stress in the presence of OVA were assessed and it was found that the treatment led to an increase in ROS, MDA, and 8-OHdG content as well as to a decrease in GSH content, indicating the production of oxidative stress (Li et al., 2014). However, there is not enough report about the comparative toxicity among these nanomaterials.

In our research, the intraperitoneal injection method was used. Sections of mice kidney were made, the organ coefficient of kidney and brain was counted, and the reactive oxygen species (ROS) level, glutathione (GSH) and malondialdehyde (MDA) content of kidney and brain was measured so as to compare different nanomaterials which may induce oxidative damage to different degrees. This study has the potential to clear the possible toxicity mechanism of different nanomaterials, and we expect our results will be able to provide some theoretical basis for evaluating the effect of the toxicity resulting from nanomaterials entering the body.

Materials and methods

Main equipment and reagents

Low-temperature refrigeration centrifuge (Eppendorf-5415R); ultrasound processor (Hielscher, Germany); FLX-800 Multi-Detection Microplate reader (Bio-Tek Instrument Inc, USA); vortex (MS1 Minishaker, IKA).

Graphene; Graphene oxide; Single wall carbon nanotubes (obtained from Nanjing XFNANO, Fig. 1 shows the characterization of graphene, graphere oxide and SWCNTs in this study); 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Sigma); trichloyoacetic acid (TCA, analytical reagent); PBS buffer (laboratory-made); 4% paraformaldehyde (laboratory-made).

Experimental animals

Twenty-eight male SPF BALB/c mice, five to six weeks old, bodyweight of 20–25 g, were purchased from the Experimental Animal Center of Hubei Province, China. All animal experiments were conducted in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Office of Scientific Research Management of Central China Normal University.

Exposure methods

G, GO and SWCNTs solution at a concentration of 0.4 mg/mL was prepared using saline solution, and then sterilized at 121°C for 30 min. They were then placed in an ultrasound processor for 3 h and subjected to ultrasound pulses 30 min before each injection to ensure that the particles were evenly distributed in solution.

Twenty-eight male BALB/c mice were randomly divided into four groups. Three experimental groups were exposed to different kinds of nanomaterials, namely graphene, graphene oxide and SWCNTs by intraperitoneal injection at exposure dosages of 4 mg/kg for 7 consecutive days. The chemicals were administered in doses of 0.01 mL/g bodyweight. The control mice were treated with saline solution. For seven consecutive days, mice were provided food and water freely. Seven days later, the mice were sacrificed by cervical dislocation.

Organ coefficient and kidney histological assay

After seven days, the mice were weighed, followed by the kidney and brain of each animal also being weighed. From this we can calculate the organ coefficient of the kidney and brain (organ coefficient = organ weight/bodyweight). Two mice from each group were used to make kidney sections. All samples were fixed in 4% paraformaldehyde solution for 24 h at −4°C, embedded in paraffin, cut into pieces, and separate pieces stained with hematoxylin and eosin (H&E), and finally

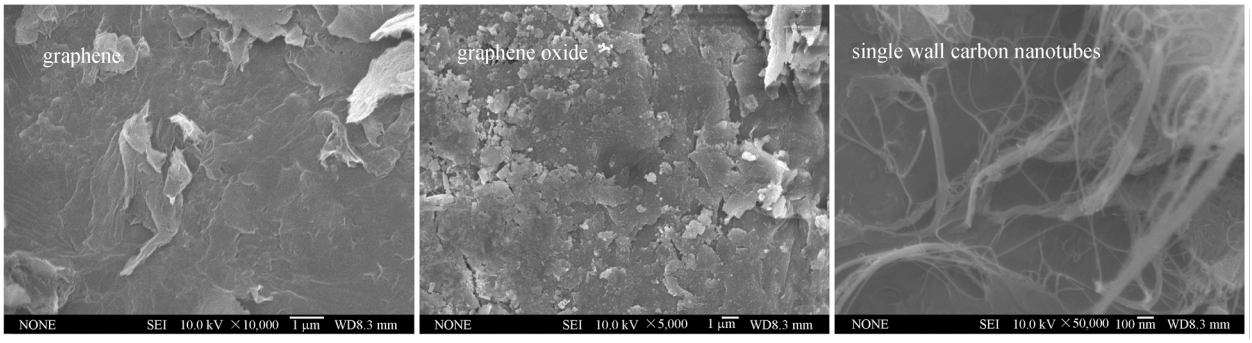

Figure 1 Scanning electron microscopy (SEM) images of graphene, graphene oxide and SWCNTs in this study.

examined using a microscope to explore the histopathological changes in the mice kidneys.

Preparation of kidney and brain tissue homogenate

The entire kidney and brain were removed from the mice using medical scissors and then rinsed in ice-cold PBS. Next the tissues were homogenized using 10 mL/g of ice-cold PBS at pH7.5 to get the tissue homogenate with a concentration of 10%. The homogenate was centrifuged at 10000 r/min at 4°C for 10 min and the supernatant collected and frozen at −70°C for later assessment of ROS, GSH, and MDA.

ROS assay

Reactive oxygen species (ROS) concentrations were determined based on the reactions between ROS and 2′,7′-dichlorofluorescein diacetate (DCFH-DA). Supernatant of kidney tissue homogenate was diluted 100-fold in PBS (2μL tissue homogenate and 198 μL PBS), while the brain tissue homogenate was diluted 20-fold (10 μL tissue homogenate and 190 μL PBS). Then 100 μL of diluted supernatant was mixed with 100 μL DCFH-DA (diluted 1000-fold with 10 μL dimethyl sulfoxide, DMSO) and placed in the wells of a 96-well microplate. The reaction mixture was kept in complete darkness at 37°C for 5min and the ROS concentration then determined using a fluorescence reader with 485 and 520 nm for excitation and emission, respectively (Crow, 1997).

GSH assay

A sample of 200 μL of tissue homogenate supernatant was mixed with 1 mL organic solvent (V (chloroform): V (butyl alcohol) = 3: 1) to precipitate all proteins present. After standing for 10 min on ice, the sample was centrifuged at 10000 r/min at 4°C for 5 min. Next a 50 μL volume of the obtained supernatant and 150 μL of 60 μg/mL DTNB solution were added to the 96-well microplate. After being kept in the dark for 5 min, the absorbance in each well was measured at 412 nm.

MDA assay

A sample of 500 μL of tissue homogenate supernatant was mixed with 2 mL of 0.6% TBA solution and incubated at 100°C for 15 min. The sample was then cooled by flowing tap water until it reached room temperature, after which it was centrifuged at 10000 r/min for 10 min. The absorbance of the sample was measured at 450, 532 and 600 nm. The following equation was used to calculate the concentration of MDA (μmol/L).

$$C = 6.45(D_{532nm} - D_{600nm}) - 0.56D_{450nm}$$

Statistical analysis

The data are presented as the mean ± SE and the statistical graphs were generated by using Origin 6.1. Results were evaluated statistically using the analysis of variance (ANOVA) followed by student's *t*-test. $p < 0.05$ was considered a significant difference and $p < 0.01$ to be an extremely significant difference.

Results

Organ coefficients in mice kidneys and brains in the different exposure groups

After 7 days' injection, Fig. 2 shows the effects of different nanomaterials on organ coefficients in mice kidneys and brains. When injected with graphene, the organ coefficients in mice kidneys and brains decreased but this decrease was not significant when compared with the control group. In the GO group, the organ coefficient in mice brains was dramatically lower than the control group ($p < 0.01$), while in the SWCNTs group, compared with the control group, the organ coefficients in both mice kidneys and brains decreased significantly ($p < 0.01$).

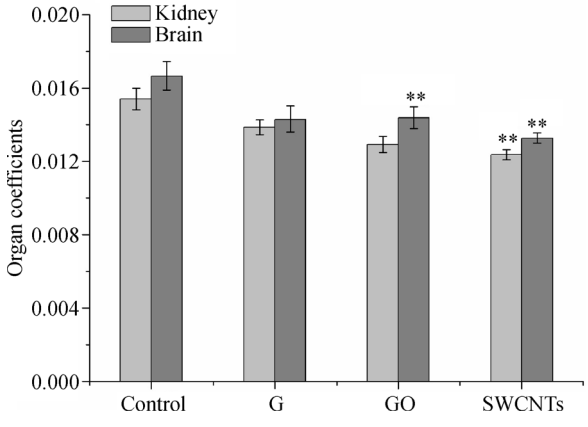

Figure 2 Organ coefficients in mice kidneys and brains of the different exposure groups.

Histopathological changes in the kidney observed by H&E staining

As shown in Fig. 3, we used the hematoxylin and eosin (H&E) staining method to reveal changes in nephric histology. No obvious abnormalities were observed in the G exposure group. The following similar changes in the kidney were observed in the GO and the SWCNTs exposure groups: renal tubular epithelial cell showed apparent swelling; the lumen of renal capsule became narrow or even disappeared; lymphocytes infiltrated the interstitial tissue of the renal

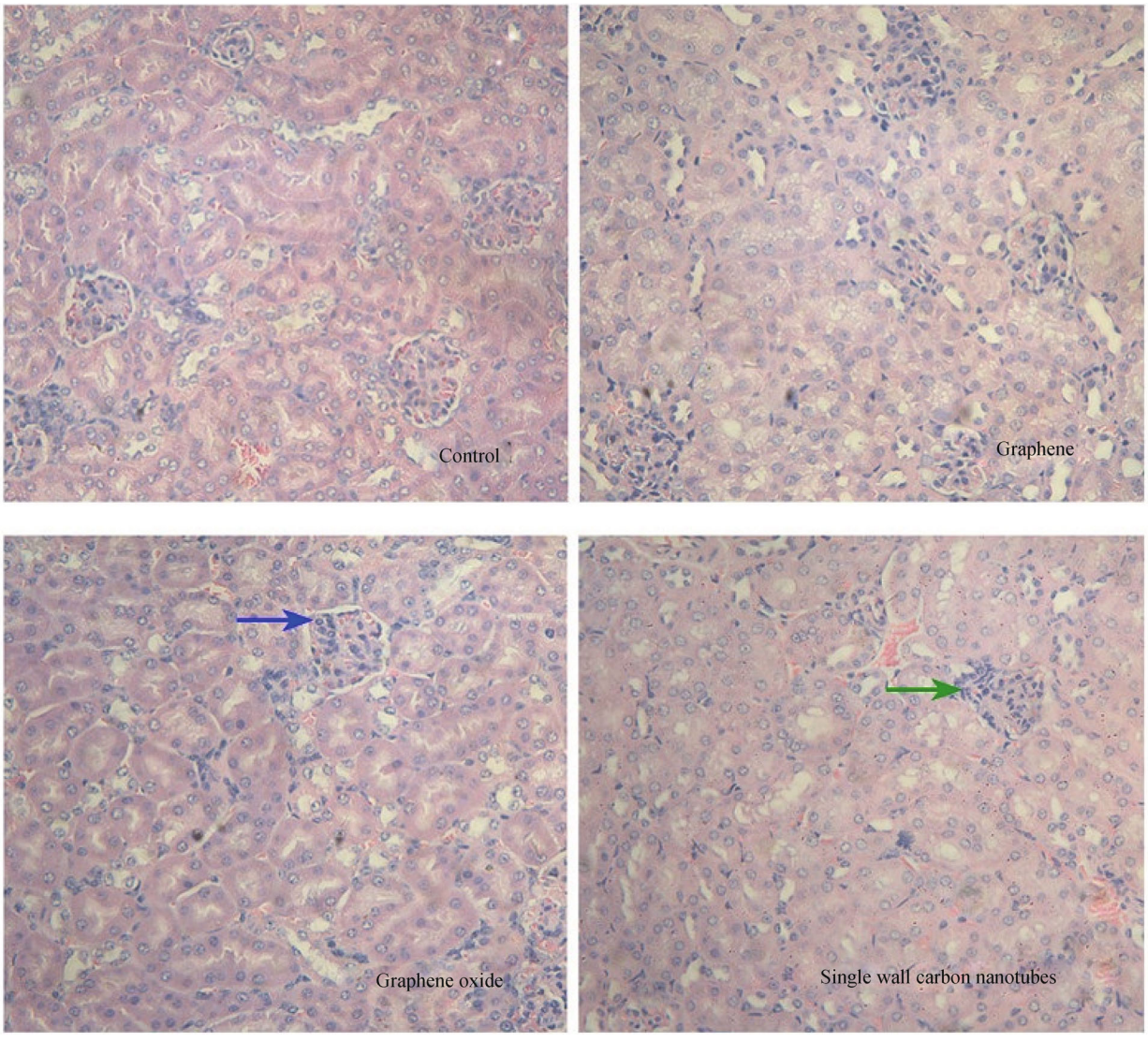

Figure 3 The pathological section diagram of a mouse kidney (20 × , H&E). Blue arrow, the lumen of renal capsule; green arrow, lymphocytes infiltration.

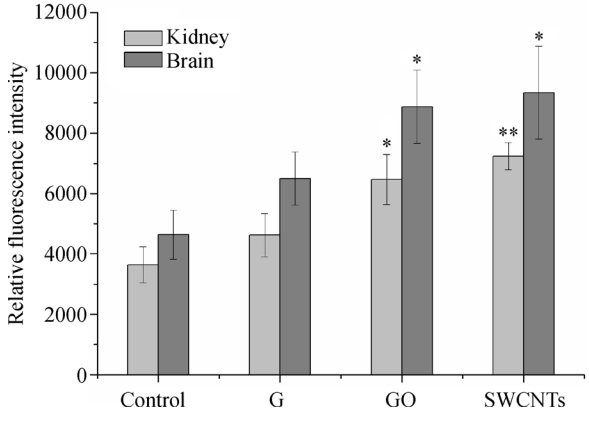

Figure 4 ROS level in mice kidneys and brains of the different exposure groups.

medulla. The histopathological changes seemed more severe in the SWCNTs exposure group.

Analysis of ROS

ROS generation increased in the different exposure groups. The ROS level in the G exposure group increased but was not obvious when compared with the control group ($p > 0.05$). ROS levels in the kidney tissue for the GO and SWCNTs exposure groups were significantly higher ($p < 0.05$ or $p < 0.01$) than the control group. In the brain cells, the generated ROS levels were also significant for the GO and SWCNTs exposure groups ($p < 0.05$).

Analysis of GSH

After 7 days' exposure, the results (Fig. 5) showed that the

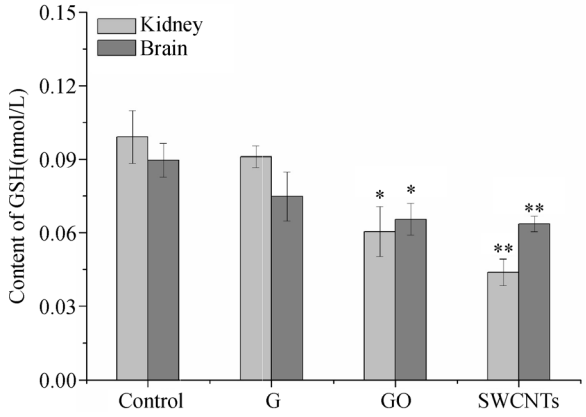

Figure 5 GSH content in mice kidneys and brains of the different exposure groups.

GSH contents in the different nanomaterials treated groups were lower than that of the saline group. However, there is no significant difference between the G exposure group and the control group ($p > 0.05$). In the GO exposure group, GSH content decreased and the decrease in this group was very significant compared with the control group ($p < 0.05$). In the SWCNTs exposure group, the GSH content in both nephric and brain cells had an extremely significant difference ($p < 0.01$).

Analysis of MDA

Figure 6 shows the difference of MDA content due to distinct treatments. MDA content in the G exposure group was almost the same as for the control group ($p > 0.05$). GO and SWCNTs caused a significant accumulation of MDA in the kidney ($p < 0.05$). In the brain cells, there was a significant difference between the GO exposure group and the control group ($p < 0.05$) and an extremely significant difference between the SWCNTs exposure group and the control group ($p < 0.01$).

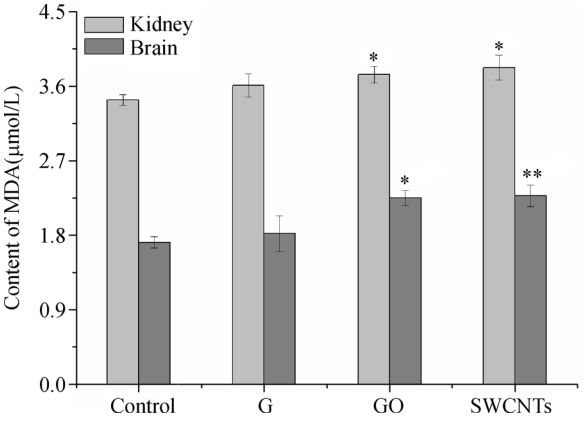

Figure 6 MDA content in mice kidneys and brains of the different exposure groups.

Discussion

Nanomaterials are highly oxidative and catalytic due to their small size but very large specific surface area (Pantarotto et al., 2004). Since it has been reported in the literature that nanomaterials can cause damage to the central nervous system through the blood-brain barrier (Kwon et al., 2008; Oberdörster et al., 2005), we chose the kidney and brain of mice to explore whether nanomaterial induced oxidative stress occurred in these organs.

The organ coefficient of a laboratory animal is the ratio of organ weight to its bodyweight. It is a sensitive indicator for subchronic toxicity testing to reflect synthetic toxicity generated by chemical poisons (Ni, 2006). The ratio is relatively constant in normal animals; however, after an animal is exposed to some toxin, the weight of the damaged organ changes and the organ coefficient will change accordingly. An increased organ coefficient suggests congestion, edema or hypertrophy of the organ, while a decrease implies viscera atrophy or other degenerative change. Therefore, from the organ coefficients of mice kidneys and brains for the different exposure groups, we can see that the damage to the kidney and brain in the G exposure group is small, in the GO exposure group, the damage to the brain is a little more severe, while in the SWCNTs exposure group, both the kidney and brain are seriously damaged.

Observing the histopathological changes in the kidney, we can see the pathological changes of mice kidney clearly so as to determine the degree of their damage directly. Renal tubular epithelial cells showed apparent swelling; the lumen of the kidney tubules narrowed or even disappeared; lymphocyte infiltration was observed in the interstitial tissue of the renal medulla. All of these changes can be seen in the GO and SWCNTs exposure groups, being more serious in the latter.

ROS is the primary indicator that reflects the level of oxygen free radicals in the cell. Under normal body conditions, ROS is found at a low level. At these levels it can't damage the cells nor the body because of the function of the antioxidant system including the antioxidant enzymes, such as super oxide dismutase (SOD), and antioxidant substances, such as, GSH (Raha and Robinson, 2000; Finkel, 2011). However, when the body is exposed to poor conditions, ROS content increases and the excessive ROS may trigger lipid peroxidation by attacking unsaturated fatty acids in the biological membrane to produce lipid peroxides, such as MDA, the content of which can reflect the intensity and speed of lipid peroxidation in the body and also indirectly reflect the extent of DNA damage (Ma et al., 2013). Sasidharan found that untreated pure graphene accumulates on the cytomembrane and causes intensive oxidative stress in cells which in turn leads to apoptosis (Sasidharan et al., 2011). In the experiment to test the cytotoxic effect of GO on hepatocytes using the MTT method, it was found that the death rate of cells increased with an increase in GO

concentration (Sun et al., 2013). Wang proved that the antioxidant systems of mice livers and kidneys may be impaired when exposed to SWCNTs (Wang et al., 2009). Our results showed that in the experimental groups, the ROS level and MDA content in mice kidneys and brains increased, whereas the GSH content decreased, when compared with the control. These results also demonstrated that oxidative damage to mice kidneys and brains nduced by SWCNTs and GO is more severe than that induced by G.

By comparing the organ coefficient and oxidative damage index, and observing the sections of mice kidneys, we see that the most toxic nanomaterial we looked at is the SWCNTs, followed by GO, while G has a relatively low toxicity. The particle size of GO and G is similar, while the chemical composition and structure are different. GO has the same planar construction as G, however it has more oxygen-containing functional groups, suggesting that the surface character and chemical modification play an important role in the differential toxicity between GO and G. The smaller the particle size of a nanomatirial, the greater the tendency to biological toxicity. Comparing the particle size of the three different nanomaterials, we see that SWCNTs are the smallest and have the greatest biotoxicity. This implies that particle size is a critical factor in the differing toxicity of these three nanomaterials. At the same time, SWCNTs can be regarded as G rolled into a cylindrical structure (flat atomic sheets for G and tubular for SWCNTs), which also implies that the toxicity is different as a result of structure. To summarize, all three of the nanomaterials tested can induce tissue damage in the kidneys and brains of mice, particularly SWCNTs and GO. Although the three materials have a similar chemical composition, the difference of their toxicity is probably related to particle size, surface character, chemical modification and structure.

Acknowledgements

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Compliance with ethics guidelines

Shuai SHANG, Shang-Yue YANG, Zhi-Min LIU, and Xu YANG declare that they have no conflict of interest. All institutional and national guidelines for the care and use of laboratory animals were followed.

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