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Effects of graphene oxide on the development of offspring mice in lactation period

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ABSTRACT

The potential toxicity of graphene oxide (GO) has attracted much attention with numerous promising biomedical applications in recent years. However, information about GO on the development of filial animals is rare. In this work, we studied the potential developmental toxicity of GO when they entered the body of maternal mice and their offspring by oral exposure with two doses. The results showed that the increase of body weight, body length and tail length of the filial mice received GO at 0.5 mg mL^{-1} (about 0.8 mg each mouse) every day in the lactation period was significantly retarded comparing with the control group. The anatomy and histology results revealed the delayed developments of offspring in high dosage group. We also evaluated the possible toxicological mechanism caused by GO and found that the length of the intestinal villus of the filial mice received high concentration GO were decreased significantly compared with the control group. It can be concluded that GO showed many negative effects on the development of mice in the lactation period. These findings can be significant for the development of graphene materials-based drug delivery system and other biomedical applications in the future.

1. Introduction

Owing to many extraordinary properties like large specific surface, flexibility, and superior electric conductivity, graphene-related materials have been increasingly employed in the fields of energy storage, biomedicine, catalysis, and so on [1–3]. Specifically, as a typical functional grahpene material, grahpene oxide (GO) have a good biocompatible property and show a great potential application in the biomedical field as drug delivery carriers, bioimaging, tissue engineering and bioassay agents due to their chemical stability, high coefficient of heat conduction, amphipathicity, extremely large surface area, and easy functionization [4–9]. Consequently, concerns on the potential toxicity caused by GO are also growing [10]. A number of studies referring to the toxicity of GO have been reported, but the potential hazard of this nanomaterial is still obscure. Some studies reported that GO was biocompatible for biomedical applications at least under the limited condition [4,11]. However, other researches reported that GO could bring about the adverse biological responses including the cytotoxicity, the acute lung injury (ALI) and the chronic pulmonary fibrosis [12,13]. Akhavan and co-workers have done many systematic works on the cyto- and geno-toxicity of graphene, and firstly proved that the toxicity of graphene was size and chemical-dependent [14,15]. Up to now, it is widely accepted that the toxicity of graphene was resulted from the direct contact interaction of sharply edged graphene with the cells and organisms [16,17], reactive oxygen species (ROS) generation [12] or wrapping the grapheme sheets around the cells and organisms [18,19]. However, to the best of our knowledge, most of these studies have been focused on investigating the short or long-term toxicity of GO exposed through difference administration routes *in vivo* [20,21], there is still no report about the reproduction toxicity of GO.

During the past decades, the reproductive and developmental toxicity has increasingly become an important part of overall nanotoxicology research [22]. As well known, newborn animals and the maternal are vulnerable subpopulation due to their special physical stage in the lactation. Particularly, the immune and intestine systems of the newborn animals is not so fully developed that they are very sensitive to the exotic substance [23]. The

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reproductive and developmental toxicity of many promising biomaterials have been investigated in detail. For example, pregnancy complications caused by silica nanomaterials have been found [24]. The toxicity studies of other nanomaterials such as multi-walled carbon nanotubes, C60, magnetic iron oxide and carbon nanoparticles on the reproductive system and the general development of offspring are also available [25–29]. However, as a kind of extraordinary and great potential material for biomedicine [9], little information is known about the potential toxicity caused by GO [30]. Thus, the toxicological and pharmacological studies of GO on the reproductive aspects are urgent and highly desirable.

In this study, both the newborn and maternal mice were used to investigate the potential toxicity caused by GO by oral exposure. The general development of offspring mice including body weight, body length, and behavior were recorded. After the offspring mice directly and indirectly drinking GO suspension for continuous 21 days, the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine (CREA), blood urea nitrogen (BUN) in the plasma, pathological changes of the main organs were also systematically evaluated. Moreover, in order to better understand the toxicity of the offspring mice, the toxicity of the maternal mice caused by GO were also evaluated through the biochemistry analysis and pathological changes. It is expected that this study will provide some basic information on the growth and development of the newborn filial mice after exposure to GO and other graphene-based nanomaterial in the lactation.

2. Materials and methods

2.1. Materials

Flake graphite was purchased from China National Pharmaceutical Group Corporation (Shanghai, China). Potassium permanganate ($KMnO_4$), potassium nitrate (KNO_3), silver nitrate ($AgNO_3$), disodium hydrogen phosphate (Na_2HPO_4), 98% sulfuric acid (H_2SO_4) solution, 30% hydrogen peroxide (H_2O_2) solution and 36–38% hydrogen chloride (HCl) were sourced from Beijing Chemical Corporation. Hematoxylin and eosin were obtained from Sigma. Other chemicals were of analytical grade. Deionized water was supplied by local sources. Female and male ICR mice, aged 8–9 weeks, were purchased from Charles River Laboratories.

2.2. Synthesis of graphene oxide (GO) nanosheets

GO was prepared by chemical exfoliation of the graphite by using a modified Hummers method referenced in previous reports [31]. In a typical experiment, 1 g flake graphite was added to 25 mL of cooled sulfuric acid. Then, 3 g of $KMnO_4$ was slowly added into above mixture under vigorous stirring with an ice bath. Afterwards, the mixture was further stirred at 35 °C for 2 h. Subsequently, 46 mL of deionized water was introduced dropwise into the above mixture, and then the temperature was gradually increased to 98 °C and maintained at that temperature for 15 min further oxidation. The reaction was stopped by slow addition of 100 mL of deionized water and 10 mL of 30% H_2O_2 solution. Finally, the product was separated by centrifugation and washed repeatedly with HCl (5 wt%) solution to remove residual potassium permanganate and sulfate until the pH value reach ~7. Afterwards, brown powders were obtained after the precipitation was dried in a vacuum oven for several days at room temperature. To obtain a colloidal suspension of individual GO nanosheets, the as-synthesized dried solid product was dispersed in water aid of ultrasound and purified with centrifugation and dialysis.

2.3. Characterization and measurements

X-ray diffraction patterns (XRD) of the samples were measured on a Bruker D8 Focus powder X-ray diffractometer using CuKα radiation ($\lambda = 1.5418$ Å). UV–visible absorption spectra were carried out using a JASCO UVvis 570UV–vis spectrophotometer in 1 cm quartz cuvettes over the range of 200–400 nm. Fourier transform infrared spectra (FT-IR) were performed on an Excalibur 3100 system (Varian) by using the KBr pellet method. X-ray photoelectron spectra (XPS) measurements were recorded on a KRATOS AXIS ULTRA/DLD spectrometer with a monochromatic AlKα radiation. Transmission electron microscope (TEM) images and selected-area electron diffraction (SAED) were taken on a JEM-2100F transmission electron microscope operated at 200 kV. The samples were prepared by depositing the GO on a carbon film coated copper grid. Atomic Force Microscope (AFM) was performed on Bruker Multimode 8 AFM in tapping mode. The samples were fabricated by dropping the GO aqueous solution on a clear mica plate. GO were dispersed in the simulated biological fluids, which were prepared according to the previous reference [32].

2.4. Animals

All animal experiments were carried out under a protocol approved by the Local Ethics Committee. 18 multifara female ICR mice with good motherhood were selected. Then, male and female mice were housed in a cage in accordance with the ratio of 1:1. After parturition, each litter was culled to 8 pups on postnatal day 1 (PND1). Afterwards, 12 mice were randomly assigned to two treatment groups that were administrated with 0.5 mg mL^{-1} and 0.05 mg mL^{-1} GO through free drinking the GO suspension dispersed in the water from PND 1 day to 21 days and one control group that was administered with normal water. On PND 21 days, a half of mice administered with 0.5 and 0.05 mg mL^{-1} GO groups were killed, 8 pups in each group were weighed and slaughter. At the same time, the remaining maternal mice and pups were administered with changing GO suspension into water, and continued feeding. They were killed on PND 38 days.

2.5. General clinical manifestation

During the experimental period, the clinical manifestations of the mice were intimately observed. The numbers of dead mice were recorded as well. At the PND 21 days and 38 days, body weight, body length and tail length of the pups were measured in detail. It needs to be mentioned that body length is referred to the length from head to trail tip and tail length is measured from the anus to the trail tip.

2.6. Blood biochemical assay

After administration for 21 days and 38 days, blood samples (about 0.8–1.0 mL) were obtained from each mouse via the orbital treating. Then the blood samples were centrifuged at 3000 rpm for 20 min to separate serum. The alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine (CREA) and aspartate aminotransferase (AST) levels of serum were tested in detail.

2.7. Histological analysis

To examine the morphological and pathological changes, the pups were slaughtered at 21 days and 38 days. The lung, liver, kidney and spleen were removed and immediately fixed in 10% formalin for further histopathological examinations. The tissues of organ samples embedded in paraffin blocks and HE staining were carried out by the standard protocol. After histological HE staining, the observation were performed using optical microscope (Nikon eclipse-Ti), and subjected to take the photos. The identity and analysis of the pathology slides were blind to the pathologist.

2.8. Statistical analysis

The results were represented by average ± standard deviation. The statistical significance of the changes between tested groups and the control group were analyzed by one way ANOVA using SPSS 17.0.

3. Results and discussion

3.1. Preparation and characterization of GO

High quality GO nanosheets were synthesized from graphite using a modified Hammer's method [31]. Transmission electron microscopy (TEM), Atomic force microscopy (AFM), UV–visible absorption spectra, Fourier transform infrared spectra (FT-IR) and X-ray diffraction (XRD) were performed to characterize the as-prepared GO. As shown in Fig. 1A, the XRD pattern of the GO exhibited two characteristic peak close to 12.0° and 43°, corresponding to the reflection of (002) and (001) with interlayer distance of 0.73 and 0.21 nm, respectively [33]. The as-obtained GO can be facilely dispersed in water for their rich oxygen-containing functional groups. The UV–vis spectra of the GO dispersion in water displayed the characteristic feature with a maximum peak at ~232 nm and a shoulder peak at ~280 nm, corresponding to $\pi-\pi^*$ transition of aromatic C–C bonds and $n-\pi^*$ transition of C=O, respectively. The FT-IR shown in Fig. 1C also revealed the stretching vibration peak of C=O at 1715 cm^{-1}, the C–O–C stretching vibration peak at 1120 cm^{-1}, and the vibration and deformation peaks of O–H at 3400 and 1620 cm^{-1}. XPS was measured to examine the chemical composition of the GO. C and O elements with a molar ratio of 2.11 were detected, suggesting the presence of abundant oxygen in the GO (Fig. S1). Besides, the C1s spectrum can be fitted into four peaks 285.0, 286.1, 287.5, and 288.5 eV (Fig. S1), which are ascribed to the graphitic (C=C), alkoxy/epoxy (C–O), carbonyl (C=