Biomaterials 54 (2015) 188–200

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Long-term toxicity of reduced graphene oxide nanosheets: Effects on female mouse reproductive ability and offspring development

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ARTICLE INFO

Article history:
Received 19 December 2014
Received in revised form
5 March 2015
Accepted 9 March 2015
Available online 11 April 2015

Keywords:
Reduced graphene oxide nanosheets
Female mouse
Reproductive toxicity
Offspring health

ABSTRACT

Reduced graphene oxide (rGO) nanosheets have emerged as novel materials for cancer therapeutics. Their toxicity has attracted much attention since these nanomaterials may have great potential for clinical cancer treatment. Here we report the influence of rGO exposure on female mouse reproductive ability and offspring development. Mouse dams were injected with small or large rGO nanosheets at different doses and time points, pre- or post-fertilization. The sex hormone levels of adult female mice did not significantly change compared with the control group after intravenous injection with either small or large rGO, even at a high dose (25 mg/kg). Mouse dams could produce healthy offspring after treatment with rGO nanosheets before pregnancy and at an early gestational stage (~6 days). Despite the successful delivery of offspring, malformed fetuses were found among rGO-injected dam litters. All mice had abortions when injected with low (6.25 mg/kg) or intermediate (12.5 mg/kg) doses at a late gestational stage (~20 days); the majority of pregnant mice died when injected with the high dose of rGO at this stage of pregnancy. Interestingly, all surviving rGO-injected mouse mothers gave birth to another litter of healthy pups. The results presented in this work are important for a deeper understanding of the toxicity of rGO nanosheets on female reproductivity and their offspring development.

1. Introduction

In recent years, graphene, a two-dimensional nanocrystalline material, has been widely used in biomedical research fields, including drug delivery [1–4], biological imaging [4–6], biosensing [7], cancer photothermal therapy [6,8–11] and tissue engineering [12,13]. Graphene oxide (GO) or reduced graphene oxide (rGO) are the most commonly derivatives because they are water-soluble and enriched with carboxylate groups on the periphery for bio-conjugation. rGO nanosheets have an extremely large ratio of area to volume and exhibit highly efficient photothermal conversion [6,8–11], meaning that they have the potential to be used as a drug delivery carrier for *in vivo* cancer combination therapy.

However, the behavior of rGO nanomaterials in the body is still not fully understood and clinical applications of rGO will not be possible until the potential toxicity to humans or animals is fully investigated. The potential toxicity of rGO in biomedical systems is therefore being extensively researched [14–23]. It has been reported that rGO can cause dose-dependent oxidative stress in A549 lung cancer cells and induce a minor loss of cell viability at high concentration [14]. rGO has also been seen to induce significant increases in both intercellular reactive oxygen species levels and in mRNA levels of hemeoxygenase 1 and thioredoxin reductase [15]. Furthermore, significant pathological changes, including pulmonary edema and granuloma formation, were found in the lungs of mice when graphene nanosheets were intravenously administered at a high dose [16]. *In vivo* toxicity results showed that rGO can remain in the mouse body for a long period of time after tail vein injection [18,19]. Therefore, concerns remain over the long-term toxicity of rGO, especially to the offspring.

In our previous work [19], we found that mouse sperm quality was not significantly influenced by exposure to graphene nanosheets. To date, there has been no investigation into the potential toxicity of this nanomaterial on female reproductive function. Here, rGO nanosheets of different sizes were administered at different doses and examined for their influence on female reproductive performance and the health of their first, second and third litters of offspring. The results of this work will be an important reference for

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http://dx.doi.org/10.1016/j.biomaterials.2015.03.015
0142-9612/

the *in vivo* toxicity of graphene nanosheets and will also provide meaningful insights on the clinical application of these nanomaterials.

2. Materials and methods

2.1. Chemicals and reagents

Exfoliated graphite flakes (~25 μm) with a purity of 99.9% were purchased from Shanghai Nan-qiu Carbon Cooperative Company (Shanghai, China). H_2SO_4, HCl, $KMnO_4$, H_2O_2 (30%), $AgNO_3$ and NaOH were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). $Na^{125}I$ was purchased from Chengdu Gaotong Isotope Co., Ltd. (Chengdu, China). Chloramine T was purchased from Sigma–Aldrich (St. Louis, MO, USA). Mouse estrogen Elisa kit was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Hematoxylin-eosin (H&E) staining kit was purchased from Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China).

2.2. Animals

Female and male ICR-strain mice (6–8 weeks old) were purchased from Shanghai Sipper-BK Lab Animal Co., Ltd. (Shanghai, China) and were used in accordance with approved institutional protocols established by the Shanghai Department of Experimental Animals Management. All mice were housed in positive-pressure air-conditioned units (25 °C, 50% relative humidity) on a 12 h light: 12 h dark cycle and provided with food and water ad libitum. The mice were acclimatized for 2 days under laboratory conditions before starting the experiments.

2.3. Synthesis of rGO

Reduced GO nanosheets were synthesized from graphite powder using a modified Hummer's method [24]; detailed protocols are shown in the Supporting Information (Supplementary Section 1: Methods).

Two sizes of rGO, 20–150 nm (small rGO) and 200–1500 nm (large rGO) were selected for the following experiments and the HEPES buffer-dispersed small and large rGO with at three different does (6.25, 12.5 and 25 mg/kg per mouse) were used for the following animal experiments. These three doses are subsequently referred to as low, medium and high dose, respectively.

2.4. Measurement of female mouse serum estrogen levels

A total of 30 adult female mice were used in these experiments. The dams were injected with 200 μL of HEPES buffer-dispersed small rGO or large rGO at the high dose or with blank HEPES buffer *via* the tail vein ($n = 10$ per group). For the 1 day and 30 days post-injection groups, five mice were anaesthetized and a ~0.8 mL blood sample from each mouse was collected from the orbital sinus. The level of estrogen was then measured using the mouse estrogen ELISA kit (R&D Systems, Inc.) according to the protocol provided.

2.5. Evaluation of reproductive ability and offspring of rGO-treated female mice

The main protocols of the following animal experiments are illustrated in Fig. 1.

2.5.1. rGO exposure methods

A total of 86 adult female mice were used in these experiments, as detailed below.

2.5.1.1. Exposure to rGO one day before mating. Female mice were randomly divided into groups and injected *via* the tail vein with 200 μL of HEPES buffer-dispersed small rGO at the low ($n = 6$), medium ($n = 5$) or high doses ($n = 9$), or with 200 μL of HEPES buffer-dispersed large rGO at the high dose ($n = 9$). One day later, the rGO-treated females were housed with untreated males. After the pregnancy of the female mice was confirmed, the males were removed and each dam was housed alone. Pups were removed from cages after four weeks of nursing.

To investigate whether rGO exhibited long-term toxicity on mouse dam reproductive capability, and whether these rGO-treated females could produce further litters of healthy offspring, rGO-treated female mice were randomly selected from the two groups treated with the high doses of rGO ($n = 4$ per group). Long-term tracking experiments were performed as follows: after rGO-treated females gave birth to the first litter of offspring, they were housed with untreated male mice for a second time at 60 days post-injection; males were removed after the females were confirmed as pregnant for a second time. The same dams were then housed with untreated male mice for a third time, at 120 days post-injection. The pups in each litter were removed from their mothers after four weeks. Untreated female mice were used as controls, either intravenously injected with 200 μL of blank HEPES buffer ($n = 5$), or not injected ($n = 4$), and the subsequent experiments were carried out as described above.

2.5.1.2. Exposure to rGO 30 days before mating. Twelve female mice were randomly divided into three groups ($n = 4$ for each group). The mice were injected with 200 μL of HEPES buffer-dispersed small and large rGO at the high doses *via* the tail vein. All groups were then housed with untreated male mice from 30 days post-injection. As a control, 200 μL of blank HEPES buffer was intravenously injected into four female mice, which were then housed with males from 30 days post-injection.

2.5.1.3. Exposure to rGO at early and late stages of gestation. Fourteen pregnant female mice were randomly assigned to three groups ($n = 5$ for each group). The mice were injected with 200 μL of HEPES buffer-dispersed small or large rGO ($n = 5$ for each group) at the high doses *via* the tail vein, at ~6 days of gestation; mice injected with 200 μL blank HEPES buffer ($n = 4$) were used as controls.

Twenty-two pregnant female mice were randomly divided into four groups. The mice were injected with 200 μL of HEPES buffer-dispersed small rGO at the low ($n = 5$), medium ($n = 5$) or high dose ($n = 7$) *via* the tail vein at ~20 days of gestation. The remaining five mice were injected with 200 μL of blank HEPES buffer, acting as a control. The surviving mouse dams were able to be cohoused with male mice again, producing second and third litters.

2.5.2. Female mouse reproductive ability and parameters measured in pups

The mating behavior, pregnancies, number of pups per litter and sex ratio of females to males were recorded. The survival ratios of pups were recorded seven days after birth. The pup body weights were recorded at 10–24 h, and at 7, 14 and 21 days after birth. Photographs of mouse pups and their mothers were also taken at those time points using a digital color camera (Coolpix 4300, Nikon, Tokyo, Japan).

2.6. Blood analysis

A total of 88 adult female mice were used in these experiments, as detailed below.

2.6.1. rGO exposures

Forty female mice were injected with 200 μL of HEPES buffer-dispersed small rGO at one of three doses (low, medium or high), or with large rGO at a high dose, or with blank HEPES buffer ($n = 8$ per group) *via* the tail vein 1 day before cohabitation.

Twenty four female mice were injected with 200 μL of HEPES buffer-dispersed small or large rGO at the high dose, or with blank HEPES buffer ($n = 8$ per group), *via* the tail vein 30 days before cohabitation.

Twenty four pregnant female mice were injected with 200 μL of HEPES buffer-dispersed small or large rGO at the high dose, or with blank HEPES buffer ($n = 8$ per group), *via* the tail vein at ~6 days of gestation.

2.6.2. Blood extraction and analysis

Four mouse dams from each of the above groups were randomly selected 2 days after giving birth to pups for a blood chemistry test and complete blood panel analysis. Approximately 0.8 mL of blood was collected per mouse.

The remaining four mouse dams in each group mentioned above were allowed to live alone with their pups for 30 days. One pup (30 days old) was then randomly selected from each litter for blood analysis (~0.8 mL per pup). Four mouse dams injected with small or large rGO or HEPES buffer one day before cohabiting with males were housed with untreated male mice for the second time 60 days post-injection and gave birth to a second litter of offspring. The same dams were then housed with untreated male mice for a third time, 120 days post-injection, and gave birth to a third litter of offspring. Blood from the second and third litters of offspring was collected at 30 days of age, using the method described above.

The serum chemistry data and complete blood panel were measured at the Shanghai Research Center for Biomodel Organism.

2.7. Analysis of malformed mouse fetuses and placentas

2.7.1. rGO exposures

A total of 55 adult female mice were used in these experiments, as detailed below.

Fifteen female mice were injected with 200 μL of HEPES buffer-dispersed small or large rGO at the high dose, or with HEPES buffer alone ($n = 5$ for each group) *via* the tail vein 1 day before cohabitation with males.

Fifteen female mice were injected with 200 μL of HEPES buffer-dispersed small or large rGO at the high dose, or with blank HEPES buffer ($n = 5$ for each group), *via* the tail vein 30 days before cohabitation with males.

Twenty five pregnant female mice were injected with 200 μL of small rGO at one of three doses (low, medium or high), with large rGO at the high dose, or with HEPES buffer alone ($n = 5$ for each group) *via* the tail vein at ~6 days of gestation.

2.7.2. Observation and recording of malformed mouse fetuses and placentas

All rGO-treated and HEPES buffer-treated parturient mice were anesthetized *via* tail vein injection of choral hydrate (4%). Fetuses and their corresponding placentas were then carefully extracted from the parturient mice by cesarean section. All fetuses and placentas were carefully examined and observations recorded.

2.7.3. Histological examination

One placenta was randomly selected from each of the above groups, fixed in 4% paraformaldehyde at 4 °C, over 24 h, embedded in paraffin, sliced into 5 μm sections