5-Day repeated inhalation and 28-day post-exposure study of graphene

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Abstract

Graphene has recently been attracting increasing attention due to its unique electronic and chemical properties and many potential applications in such fields as semiconductors, energy storage, flexible electronics, biosensors and medical imaging. However, the toxicity of graphene nanomaterials during manufacturing and use related to exposure to graphene. This is particularly relevant to impact on human health, for example, pulmonary diseases widespread use has also raised some concerns over the potential toxicity from the results presented in previous studies. Therefore, these results suggest that the 5-day repeated exposure to graphene only had a minimal toxic effect at the concentrations and time points used in this study.

Introduction

Graphene is a recently developed nanomaterial that possesses unique physicochemical properties, and thus a number of potential applications are expected in many areas of industry and science, such as electronics, pharmaceutics and medicine (Zhu et al., 2010). More specifically, graphene nanomaterials have been suggested for use in transistors, sensors, films, clean energy devices, DNA sensing and drug delivery (Balapanuru et al., 2010; Zhang et al., 2010; Zhu et al., 2010). However, this possible widespread use has also raised some concerns over the potential impact on human health, for example, pulmonary diseases related to exposure to graphene. This is particularly relevant to the case of occupational and environmental inhalation exposure to graphene-based nanomaterials during manufacturing and use (Schinwald et al., 2012).

Yet, only a small number of studies have so far reported on the pulmonary effects of graphene nanomaterials. Among the results, graphene oxide has been found to induce cytotoxicity and genotoxicity in human lung fibroblast cells in a dose-dependent manner (Wang et al., 2013), where oxidative stress and the surface charge of graphene oxide have been shown to mediate the toxicity. The toxicity of graphene has also been demonstrated in in vivo and in vitro experimental settings, where graphene nanoplates were found to induce the expression of inflammatory cytokines in mouse lungs after a single instillation (Park et al., 2014) and cause reduced cell viability, mitochondrial damage and elevated autophagosome-related proteins in a human bronchial epithelial cell line, BEAS-2B (Park et al., 2014). In addition, graphene oxide has also been shown to increase cytotoxicity and apoptosis in BEAS-2B cells (Vallabani et al., 2011). Notwithstanding, other in vitro data have shown that low doses of graphene oxide did not induce cytotoxicity in lung epithelial cells, A549 (Chang et al., 2011). Finally, an in vitro study of graphene nanoplatelets observed inflammatory effects in mouse lungs, where the pharyngeal aspiration of graphene nanoplatelets increased the expression of proinflammatory cytokines in the bronchoalveolar lavage and pleural lavage (Schinwald et al., 2012). However, since the abovementioned studies only employed intratracheal instillation or a pharyngeal aspiration technique in an animal model, the results are limited as regards reflecting the real inhalation situation in an occupational or environmental setting, meaning that the exposure of animals to graphene could produce different toxic responses from the results presented in previous studies.

Accordingly, this study evaluated the toxic effects of graphene based on a short-term inhalation study (i.e. 28-day study with a 5-day inhalation exposure of animals) using a nose-only inhalation system for the collection of initial toxicity data on nanomaterials (Ma-Hock et al., 2009). In addition, this short-term animal study will confirm whether a long-term animal study is also needed for testing the nanomaterials (Klein et al., 2012). Thus, the animals were exposed to a graphene nanopowder in a nose-only inhalation system for 5 days and then allowed to

Keywords

Nanomaterials, nanoparticles, nanotoxicology, particle toxicology, toxicity
recover for 1, 3, 7 or 28 days. The resulting toxicity information provides the necessary data for a safety evaluation of the graphene nanopowder used in this study.

**Materials and methods**

**Characterization of graphene nanopowder**

The graphene nanopowder (average flake thickness, 8 nm; average lateral size, ~550 nm; surface area, 100 m²/g; purity, 99.9%) was provided by Graphene Supermarket (Calverton, NY). A transmission electron microscope equipped with an energy dispersive X-ray analyzer (FE-TEM, JEM2100F, JEOL, Japan) was used to measure the graphene nanopowder based on National Institute for Occupational Safety and health (NIOSH) analytical method 7402 (NIOSH 1994). The graphene nanopowder was mounted on a TEM grid (copper grid) and visualized under a field emission-transmission electron microscope (FE-TEM, JEM2100F, JEOL, Japan). The nanopowder was measured at a magnification of 100,000 and analyzed using an energy-dispersive X-ray spectrometer (EDS, TM200, Oxford, UK) at an accelerating voltage of 75 kV.

**Aerosol generation**

Sprague-Dawley (SD) rats were exposed to the graphene nanopowder using a nose-only exposure system (HCT, Incheon, Korea). The graphene nanopowder was generated using an atomizer (AG-01, HCT, Icheon, Korea) (Supplementary data S1), and purified air was used as the carrier gas. The suspension contained 50 mg/ml for the high concentration, and 5 mg/ml for the low concentration. The gas flow was maintained at 301 per minute (l/min) using a mass flow controller (MFC, AERA, FC-7810CD-4 V, Tokyo, Japan), and the flow rate to each nose port was 11/min. The AC power supply was maintained at 99.56 ± 0.07 V (mean ± SE). The target concentrations of the generated graphene nanopowder were 0.5 and 3 mg/m³ for the low and high concentration, respectively. The mass median aerodynamic diameter (MMAD) was measured using a DLPI (Dekati® Low Pressure Impactor, Kangasala, Finland), which is a 13-stage cascade low-pressure impactor that can determine the particle geometric standard deviation (GSD) was then obtained using an energy-dispersive X-ray spectrometer (EDS, TM200, Oxford, UK) at an accelerating voltage of 75 kV.

**Animals and conditions**

The six-week-old male specific-pathogen free SD rats were purchased from OrientBio (Seongnam, Korea) and acclimated to 4°C for 2 weeks before initiating the inhalation exposure. During the acclimation and inhalation exposure, the rats were housed in polycarbonate cages (maximum of 3 rats per cage) installed in individually ventilated cage racks. The rats were kept under a controlled temperature (22 ± 0.65 °C) and humidity (56 ± 0.08%), and a 12-h light/dark cycle. The rats were fed a rodent diet (Woojung BSC, Suwon, Korea) and filtered water ad libitum. During the acclimation period, the animals were trained to adapt to the nose-only inhalation chamber for 6h/day. The rats were divided into three groups: control (unexposed, n = 20), low-concentration group (n = 20), and high-concentration group (n = 20). The low- and high-concentration groups were exposed to the graphene nanopowder for 6h/day for 5 days, while the control group received filtered fresh air. The animals were examined daily for any evidence of exposure-related toxic responses. The body weights were measured at the time of purchase, at the time of grouping, once during the inhalation period, and before necropsy. The food consumption (g/rat/day) was measured once a week. After the 5 days of graphene exposure, the rats were allowed to recover for 1, 3, 7 or 28 days (n = 5 per treatment group for each time period) to investigate the tissue toxic responses. At sacrifice, gross observations of the organs were recorded, plus the testes, kidneys, spleen, liver, lungs and brain were all carefully removed and weighed. All the animal protocols were approved by the Hanyang University Institutional Animal Care and Use Committee.

**Monitoring of inhalation chamber and analysis of graphene nanopowder**

The distribution of the graphene nanopowder with respect to size was measured directly using a scanning nanoparticle spectrometer (SNPS, HCT Co., Ltd., Icheon, Korea) connected to a condensation particle counter (CPC, model 3022A, TSI Inc., Shoreview, MN) and dust monitor (Model 1.1.09, Grimm Technologies Inc. Douglasville, GA). The graphene concentrations were measured using sheath air and poly-disperse aerosol air at 15 and 1.5l/min, respectively. The samples were collected on a polycarbonate filter from the top and bottom parts of the port using an MSA Escort ELF sampling pump (MSA, Pittsburgh, PA) at a flow rate of 1.0 l/min.

**Cumulative lung deposit dose calculation**

The daily lung burden per rat was estimated for 6h of continuous exposure, a minute ventilation of 0.19 l/min (Whalan et al., 2006), particle (MMAD 567 nm, GSD 2.4) lung deposition efficiency of 7.7% (MPPD, 2002), and graphene concentrations of 3.86 and 0.68 mg/m³. The following calculations were made (Alexander et al., 2008):

\[
\text{Graphene concentration} \times \text{minute volume} \times \text{exposure duration} \times \text{deposition efficiency} = \text{Daily deposited dose}
\]

\[
= 3.86 \text{mg/m}^3 \times (0.19 \text{ l/min}) \times 6 \text{ h} \times 0.077 = 0.0203 \text{ mg deposited per day}
\]

\[
= 0.68 \text{mg/m}^3 \times (0.19 \text{ l/min}) \times 6 \text{ h} \times 0.077 = 0.0036 \text{ mg deposited per day}
\]

Daily deposited dose \times \text{number of days} = \text{cumulative dose}

0.0203 mg/day \times 5 \text{ days} = 0.102 \text{ mg (high-concentration exposure)}

0.0036 mg/day \times 5 \text{ days} = 0.018 \text{ mg (low-concentration exposure)}

**Hematology and blood biochemistry**

At sacrifice, the rats were anesthetized with an i.p. injection of Entobar® (1 ml/kg). Blood samples were then collected from the abdominal aorta and the blood biochemistry was analyzed using a biochemical blood analyzer (Hitachi 7108, Hitachi, Tokyo, Japan). The hematology of the blood samples was also analyzed using a blood cell counter (Hemavet 0950, CDC Tech., Irvine, CA).

**Histopathology and high resolution imaging**

At sacrifice, the left lungs were removed and fixed in a 10% formalin solution containing neutral phosphate-buffered saline under 25 cm water pressure for the histopathological evaluation. Thereafter, the lungs were embedded in paraffin, stained with hematoxylin and eosin, and examined under a light microscope. In addition, the graphene imaging was examined using a high resolution illuminator (Cytoviva, Auburn, AL) attached to a darkfield microscope, as described in our previous study (Han et al, 2014).

**Bronchoalveolar lavage cell evaluation**

At sacrifice, the right lungs were lavaged 14 times with 3 ml aliquots of warm calcium- and magnesium-free phosphate
buffered saline (pH 7.4). The BAL samples were then centrifuged for 7 min at 500 × g, and the BAL cells collected and resuspended in 1 ml of phosphate-buffered saline for evaluation. Total cell number was determined using a hemocytometer. The cells were first smeared and then stained with Wright Giemsa Sure Stain to allow a count of the total number of cells, macrophages, polymorphonuclear cells (PMNs) and lymphocytes. Two hundred cells were evaluated for cell differentiation. The BAL levels of micro-albumin and lactate dehydrogenase (LDH) were also measured using a blood biochemical analyzer (Hitachi 7108, Hitachi, Japan), and the BAL fluid oxidative stress tested by measuring the concentration of hydrogen peroxide and malondialdehyde (MDA).

Statistical analysis
The statistical analysis was performed using SPSS (Version 19, Chicago, IL). The statistical evaluation was performed using an analysis of variance (ANOVA) following multiple comparison tests using Duncan’s method. The level of statistical significance was set at $p < 0.05$ and $p < 0.01$.

Results

Characteristics of graphene nanopowder
The field emission TEM analysis of graphene revealed that the graphene nanopowder was characterized as a stacked platelet structure, and showed various thicknesses following aerosol generation, ranging from 0.39 to 9.24 nm (Figure 1A–C). Also, the TEM-EDS analysis indicated the presence of four elements (i.e. C, O, Na and P) (Figure 1D). Table 1 shows the major graphene components in atomic percentages based on the EDS analysis: carbon (84.42%), oxygen (8.55%), sodium (6.02%) and phosphorus (1.0%).

Monitoring chamber and graphene distribution
The temperature, humidity and differential pressure were 23.85 ± 0.01 °C, 48.94 ± 0.15%, and −76.15 ± 0.25 Pa, respectively, for the low-concentration exposure chamber and 24.27 ± 0.02 °C, 45.4 ± 0.21%, and −78.63 ± 0.35 Pa, respectively, for the high-concentration exposure chamber. The particle concentrations (particles/cm$^3$) in the chambers were measured directly using SNPS and were $1.88 \times 10^5 \pm 1.07 \times 10^4$ and $9.97 \times 10^5 \pm 8.99 \times 10^4$ for the low-dose and high-dose of graphene, respectively (Table 2). The OPC particle count (count/l) for the low-dose and high-dose was $6.98 \times 10^4 \pm 8.60 \times 10^3$ and $2.16 \times 10^5 \pm 1.32 \times 10^4$, respectively (Table 2). The graphene exposure concentration measured based on weighing the polycarbonate filter before and after sampling was well-maintained, and the mean concentrations of graphene

| Element | Weight% | Atomic% |
|---------|---------|---------|
| C       | 76.80   | 84.42   |
| O       | 10.36   | 8.55    |
| Na      | 10.49   | 6.02    |
| P       | 2.36    | 1.00    |
| Total   |         | 100.00  |

Figure 1. Analysis of graphene with FE-TEM (A–C) (Field emission-transmission electron microscope) (×100,000), (D) EDS-spectrometer.
(mg/m³) were 0.68 ± 0.14 and 3.86 ± 0.94 for the low- and high-dose groups, respectively (Table 2, Figure 2). The size distribution and number of graphene particles in the chamber were also measured during the exposure period (6 h/day for 5 days) using SNPS (Figure 2). The particle size ranged from 10 to 130 nm, with the highest peak at 50 nm and 80 nm for the low and high concentration, respectively. Also, the MMAD and GSD for the graphene in the aerosols were 567 nm and 2.4, respectively (Figure 3). The maintenance of the particle number concentrations is shown in Figure 4.

Table 2. Distribution of graphene nanoparticles in exposure chamber.

| Group | Number by SNPS (particles/cm³) | OPC (count/l) | Mass concentration (mg/m³) |
|-------|--------------------------------|--------------|---------------------------|
| Control | 3.73 ± 2.35                  | 3.73 ± 5.17    | 0.03 ± 0.03               |
| Low    | 1.88 ± 1.07 × 10⁴          | 6.98 ± 8.60 × 10³ | 0.68 ± 0.14               |
| High   | 9.97 ± 8.99 × 10⁴           | 2.16 ± 1.32 × 10⁴ | 3.86 ± 0.94               |

Animal observation, food consumption, and effect on body and organ weights

No significant gross effects were observed during the exposure and recovery periods. Also, there were no significant differences in food intake between the control and graphene-treated groups, except for week 1 during the graphene inhalation, when the control and low-dose groups consumed a significantly larger amount of food (Supplementary data S2). However, the food intake was similar between the groups during the recovery period. Significant body weight losses ($p < 0.01$) were recorded for the low-dose and high-dose groups after the 5-day exposure, 3-day recovery and during week 2 (Table 3). No other significant body weight changes were noted at the remaining observation times.
Figure 4. Particle number concentration of graphene during 5 days of exposure measured by SNPS.

| Days | Concentration, #/cc |
|------|---------------------|
| 0    | 2.0e+5              |
| 1    | 4.0e+5              |
| 2    | 8.0e+5              |
| 3    | 1.0e+6              |
| 4    | 1.2e+6              |
| 5    | 1.4e+6              |

Effects on hematology and blood biochemistry

Among the 20 hematology parameters measured in this study, only a small number of markers showed significant changes as a result of graphene exposure. The hematological tests of the rat blood revealed significant changes in the neutrophil, lymphocyte and monocyte counts for the high-dose group when compared with the control after the 5-day exposure and 1-day recovery (Table 4). The decrease in the weight of the right kidney was observed for the high-dose group after the 28-day recovery period (i.e. control, 0.38 ± 0.01 g versus high-dose, 0.34 ± 0.01 g). In addition, a decrease in the weight of the right kidney was observed for the high-dose group after the 28-day recovery period (i.e. control, 0.38 ± 0.01 g versus high-dose, 0.34 ± 0.01 g).  

Table 3. Body weights of rats exposed to graphene for 5 days followed by 28 days of recovery.

| GROUP: (mean ± S.E) | UNIT: g | SEX: MALE |
|---------------------|---------|-----------|
| Control             | Low     | High      |
| 0 Day               | 238.01 ± 1.93 (20) | 238.47 ± 1.95 (20) | 238.94 ± 1.96 (20) |
| exposure before     | 270.52 ± 2.18 (20) | 265.05 ± 5.68 (20) | 270.20 ± 2.14 (20) |
| Sacrifice (5-day exposure) | 264.82 ± 2.30 (5) | 251.56 ± 1.59a (5) | 249.28 ± 4.09a (5) |
| Sacrifice (3-day recovery) | 304.66 ± 2.72 (5) | 275.56 ± 6.29a (5) | 272.52 ± 5.53a (5) |
| 2nd week            | 311.03 ± 2.76 (10) | 292.05 ± 2.52a (10) | 292.54 ± 2.64a (10) |
| Sacrifice (7-day recovery) | 342.10 ± 5.48 (5) | 330.86 ± 5.31a (5) | 332.24 ± 4.72a (5) |
| 3rd week            | 362.24 ± 7.54 (5) | 348.52 ± 4.33 (5) | 344.34 ± 3.73 (5) |
| 4th week            | 385.08 ± 8.77 (5) | 392.72 ± 2.10 (5) | 383.48 ± 4.83 (5) |
| 5th week            | 422.62 ± 9.48 (5) | 430.12 ± 3.79 (5) | 393.24 ± 14.53 (5) |
| Sacrifice (28-day recovery) | 417.86 ± 9.28 (5) | 426.94 ± 6.23 (5) | 411.32 ± 12.22 (5) |

(): number of animals.

*p<0.01: control versus other groups.

When examining the rat organs, including the testes, kidneys, spleen, liver, lungs (left) and brain, no significant clinical signs or organ weight changes were found at any of the observation times, with only a minor exception (Supplementary data S3–S6). For example, a significant increase in the brain weight was found for the low-dose group after the 3-day recovery period (i.e. control, 0.66 ± 0.01 g versus low-dose, 0.73 ± 0.01 g). In addition, a decrease in the weight of the right kidney was observed for the high-dose group after the 28-day recovery period (i.e. control, 0.38 ± 0.01 g versus high-dose, 0.34 ± 0.01 g).

Effects on BAL fluid

To measure the oxidative stress, the hydrogen peroxide level was determined in the BAL fluid from the rat lungs. The inhalation of graphene only increased the production of hydrogen peroxide in the low-dose and 7-day recovery groups when compared with the control (p<0.05, Table 5). No other significant alteration in the hydrogen peroxide levels was observed at the other time points when compared to the control (Table 5). In addition, the MDA levels were not significantly changed in the graphene-exposed rats when compared to the control (Table 5). When measuring the LDH levels in the BAF fluid to determine the lung cell damage, no statistically significant changes were found between the groups in all the time points tested (Table 5). The micro-albumin levels in...
Table 4. Hematology of rats after 5 days of graphene exposure, followed by 1, 3 and 28 days of recovery.

| GROUP                        | (mean ± S.E) | Unexposed | Low            | High           |
|------------------------------|--------------|-----------|----------------|----------------|
| 5 days exposure/1 day recovery |              |           |                |                |
| NE (×10³/µl)                 | 0.63 ± 0.07  | 1.15 ± 0.18 | 0.84 ± 0.10    |
| LY (×10³/µl)                 | 4.08 ± 0.36  | 6.10 ± 0.58 | 6.57 ± 1.15    |
| MO (×10³/µl)                 | 0.06 ± 0.01  | 0.08 ± 0.01 | 0.07 ± 0.01    |
| 5 days exposure/3 days recovery |            |           |                |                |
| WBC (×10³/µl)                | 7.12 ± 0.44  | 5.95 ± 0.40 | 4.51 ± 0.86    |
| LY (×10³/µl)                 | 6.23 ± 0.40  | 5.05 ± 0.35 | 3.93 ± 0.72    |
| 5 days exposure/28 days recovery |           |           |                |                |
| MCHC (g/dl)                  | 31.84 ± 0.20 | 32.48 ± 0.20 | 32.98 ± 0.25   |

Table 5. Concentrations of hydrogen peroxide (H₂O₂), malondialdehyde (MDA), lactate dehydrogenase (LDH) and microalbumin (MA) in BAL fluid.

| GROUP                          | (mean ± S.E) | 1 Day | 3 Days | 7 Days | 28 Days |
|--------------------------------|--------------|-------|--------|--------|---------|
| Control                        |              |       |        |        |         |
| H₂O₂ (µM/l)                    | 0.63 ± 0.21  | 0.94 ± 0.10 | 0.76 ± 0.05 | 1.25 ± 0.13 |
| MDA (µM/l)                     | 46.79 ± 8.43 | 104.45 ± 9.77 | 59.46 ± 1.89 | 53.91 ± 2.27 |
| LDH (u/l)                      | 22.00 ± 0.91 | 33.00 ± 0.20 | 24.00 ± 0.96 | 40.00 ± 0.52 |
| MA (u/l)                       | 0.53 ± 0.11  | 0.89 ± 0.07 | 0.67 ± 0.04 | 1.27 ± 0.09 |
| Low                            |              |       |        |        |         |
| H₂O₂ (µM/l)                    | 0.55 ± 0.19  | 0.95 ± 0.10 | 0.97 ± 0.06  | 0.86 ± 0.14 |
| MDA (µM/l)                     | 81.33 ± 1.41 | 71.44 ± 8.57 | 60.64 ± 3.76 | 55.42 ± 2.60 |
| LDH (u/l)                      | 22.00 ± 0.61 | 31.00 ± 1.57 | 24.00 ± 0.02 | 35.00 ± 0.87 |
| MA (u/l)                       | 0.56 ± 0.14  | 0.87 ± 0.09 | 0.91 ± 0.00  | 1.09 ± 0.11 |
| High                           |              |       |        |        |         |
| H₂O₂ (µM/l)                    | 0.87 ± 0.04  | 0.71 ± 0.08 | 0.75 ± 0.02 | 0.85 ± 0.16 |
| MDA (µM/l)                     | 74.82 ± 7.29 | 77.55 ± 9.58 | 80.31 ± 4.05 | 95.81 ± 31.02 |
| LDH (u/l)                      | 32.00 ± 0.13 | 28.00 ± 0.85 | 22.00 ± 0.69 | 21.00 ± 0.48 |
| MA (u/l)                       | 0.75 ± 0.07  | 0.73 ± 0.14 | 0.69 ± 0.03 | 0.87 ± 0.15 |

*p<0.05, control versus Low.

Histopathology and high resolution imaging

The histopathological examination of the rat lungs did not reveal any specific changes in the low-dose group after the 5-day exposure and 1-day recovery (Figure 5). However, a slight thickening of the alveolar wall was found in three of the five rats in the high-dose group at the same time point (Figure 5F). Also, alveolar macrophages with ingested graphene were visualized in the high-dose group (Figure 5F). After 3 days recovery, thickening of the alveolar wall was found in three of the five rats in the high-dose group, yet this was reduced to one of the five rats after 7 days recovery (Figure 6). No significant pathological changes were found in either the low- or the high-dose group after 28 days recovery (Figure 6). When the lung tissue was further analyzed using high resolution imaging, graphene was mostly visualized in the alveolar macrophages for both the low- and high-dose groups after the 5 days of graphene inhalation and 1, 7 and 28 days of recovery (Figure 7).

Discussion

The aim of this study was to evaluate the toxic effects of graphene nanomaterials, particularly graphene nanopowder, which was characterized as a stacked platelet structure with a thickness of 9.24 nm and individual platelet thickness of 0.39 nm. As this strong and light nanomaterial is expected to be used in many areas of industry, including electronics, energy and sensors, there are many concerns about human exposure in environmental and occupational settings (Sanchez et al., 2012).

Although human exposure to nanomaterials can occur through multiple routes, including inhalation, ingestion, skin absorption, and injection or implantation, the major route of human exposure to nanomaterials, such as graphene is through inhalation (Sanchez et al., 2012). Therefore, a short-term inhalation study (5-day inhalation exposure and 28-day post-exposure) was conducted, as previously reported by the current authors, as the initial step for a hazard assessment (Han et al., 2014). The toxicity information from this short-term study was found to be comparable to the results from more extensive sub-chronic inhalation studies (Klein et al., 2012; Ma-Hock et al., 2009; OECD, 2010). For example, the pulmonary effects resulting from short-term inhalation of nano-titanium dioxide were similar to those previously reported in subchronic inhalation studies (Ma-Hock et al., 2009). In the current study, male rats were exposed to two different concentrations of graphene (low-dose, 0.68 ± 0.14 mg/m³ versus high-dose, 3.86 ± 0.94 mg/m³) in order to observe the dose-dependent toxic responses in animals.

Overall, no significant clinical changes due to graphene exposure were observed during the 5-day inhalation and 28-day recovery period. A decrease of body weight was recorded for the low- and high-dose graphene inhalation groups following the 5-day exposure and at the early recovery time points up to the second week. This reduced body weight gain was likely related to the food intake, as a reduced food intake was observed for the low- and high-dose groups up to the second week. Plus, exposure-related stress was another contributing factor, as the control group also showed a reduced food intake and body weight gain up to the second week. For the longer recovery time (after the second week), the body weight and food consumption both returned to normal in the control and graphene-treated groups. There was also no significant change in the weights of most organs following the graphene inhalation, although minor exceptions were noted without any dose-dependency. Consequently, these clinical observations suggest that the inhalation of the selected concentrations of graphene only presented a minimal effect on the exposed rats.

Various other factors were also evaluated to determine the toxicity of the nanomaterials in the exposed animals. Hematological tests for 20 markers in the blood revealed no significant changes for most of the markers, although minor changes were found in the neutrophils, lymphocytes, monocytes, white blood cells, and mean corpuscular hemoglobin concentration. After 1 day of recovery, the neutrophils and monocytes showed no dose-dependency, yet the lymphocytes showed a dose-dependent increase and significantly higher levels in the high-dose group. The high-dose group also showed significant increases of certain markers, such as the white blood cells and lymphocytes after 3 days of recovery and the mean corpuscular hemoglobin concentration after 28 days of recovery. Such results indicate that the high-dose of graphene (3.86 ± 0.94 mg/m³) was somewhat associated with immunological responses in the blood.
Figure 5. Lung histopathology after 5 days of graphene exposure followed by 1 day recovery. (A) control 100×; (B) control 400×; (C) low-dose 100×; (D) low-dose 400×; (E) high-dose 100×; (F) high-dose 400×. Arrows indicate alveolar wall thickness and macrophage phagocytosis of graphene, respectively.

Figure 6. Lung histopathology after 5 days of graphene inhalation followed by 3, 7 and 28 days recovery. Control, A, D, G; Low, B, E, H; High, C, F, I. Arrows indicate increased alveolar wall thickness.
Notwithstanding, since most of the hematology markers were not markedly altered following the graphene exposure, it would appear that graphene inhalation at the selected concentrations is not toxicologically relevant. Similarly, the serum biochemical values for 22 markers supported the previously observed minimal toxicity obtained from the hematological test. The BAL fluid measurements were also failed to show any apparent toxic effects linked to the inhalation of graphene. The toxicity indicators from the BAL fluid, including the hydrogen peroxide level, LDH, micro-albumin and BAL cells, were not markedly modulated by the graphene inhalation. Similar results about minimal oxidative stress and inflammatory responses due to graphene exposure of mice lungs have also been previously reported (Schinwald et al., 2014), where the pharyngeal aspiration of un-oxidized multi-layered graphene platelets showed minimal inflammation and oxidation after 6 weeks without graphene degradation in the lung tissues. The inhalation toxicity of graphene was also recently investigated using a short-term inhalation method (6 h/d, for 5 days) and doses of 0.54, 3.05 and 10.1 mg/m³ (Ma-Hock et al., 2013). While the total cell counts, lymphocytes, PMN and eosinophil counts were all increased with a dose of 10.1 mg/m³, most of the BAL fluid toxicity parameters were not elevated at 0.54 and 3.05 mg/m³, except for PMN with a dose of 3.05 mg/m³. Overall, these results are similar to the present data at similar graphene concentrations (i.e. 0.54 versus 0.68 mg/m³ and 3.05 versus 3.86 mg/m³), where graphene induced minimal toxic responses and no organ weight changes in the male rats. Notwithstanding, a recent study showed that the instillation of graphene in mice increased pro-inflammatory cytokines, such as interleukin-1β, tumor necrosis factor-α and interleukin-6 and anti-inflammatory transforming growth factor-β, during the observation period (up to 28 days) after a single exposure to graphene (2.5 mg/kg and 5.0 mg/kg) in a dose-dependent manner (Park et al., 2014). However, this same study also found that graphene down-regulated the production of reactive oxygen species, while reducing cell viability in bronchial epithelial cells, BEAS-2B. In another previous study, a single pharyngeal aspiration of graphene (50 μg/mouse) produced elevated inflammatory responses in mouse lungs, including an increase in the polymorphonuclear leucocytes, total cell number, LDH and pro-inflammatory cytokines (MCP-1, MIP-1α, MIP-1 and IL-1β) at 24-h post exposure (Schinwald et al., 2012). In addition, the graphene exposure also showed granulomatous lesions in the bronchiole lumen and near the alveolar regions. The dose of 50 mg graphene (approximately 2.5 mg/kg mouse) in this previous study is much higher than the doses used in the present study (approximate deposited dose of 0.102 mg (0.385 mg/kg rat) for the high-concentration group assuming a deposition rate of 7.7%). Thus, when comparing the two studies, the lack of toxic responses in the current data may imply that the deposited dose by inhalation was much less than the aspiration dose. Since there is limited research data about graphene inhalation and pulmonary responses using animals, a direct comparison of the current results with other previous reports was limited. This may be due to many differences in experimental design, such as the type of animal and graphene, exposure methods and doses, meaning that the toxic responses of animals to graphene can vary and require further studies.

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Similar to MWCNTs, which have been reported to show bioptipersistence in the lungs following inhalation (Kim et al., 2012, 2014; Mercer et al., 2013), the graphene inhalation exposure also exhibited biopersistence in the lungs after the 28-day recovery. However, while MWCNT structures have been found in macrophages, lymph nodes, the lung parenchyma and plural region, the
graphene was mostly observed in the lung macrophages. Therefore, the present results indicate that the graphene nanomaterial used in this study showed a similar biopersistence to MWCNTs in rat lungs, yet the clearance and translocation of these nanomaterials would seem to behave differently. Although the current histological examination of the rat lungs showed a slight thickening of the alveolar wall and the ingestion of graphene by alveolar macrophages in some of the rats in the high-dose group after 7 days of recovery, only minimal pathological changes were observed in the lung tissues over the recovery period. Notwithstanding, in the present study, high-resolution dark-field imaging revealed graphene deposition in the lungs, mostly in the alveolar macrophages, in the samples from both the low- and high-dose groups over the recovery period. In a previous report, which also found a deposition of graphene in mouse lung tissue, there was no degradation of the nanomaterial or tissue deposition for 6 weeks following the lung exposure (Schinwald et al., 2014). This previous study was also found that the pharyngeal aspiration of graphene in mice-only induced minimal inflammation after 6 weeks, despite the observation of large deposit of graphene.

Thus, when taken together, the results of this study suggest that the inhalation of graphene by male rats had a minimal effect on the lungs and other organs, however, further studies with varying experimental settings may be useful.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Supplementary material available online

Supplementary data S1–S13