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Short-term inhalation study of graphene oxide nanoplates

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Abstract

Graphene oxides possess unique physicochemical properties with important potential applications in electronics, pharmaceuticals, and medicine. However, the toxicity following inhalation exposure to graphene oxide has not yet been clarified. Therefore, this study conducted a short-term graphene oxide inhalation toxicity analysis using a nose-only inhalation exposure system and male Sprague-Dawley rats. A total of four groups (15 rats per group) were exposed: (1) control (fresh air), (2) low concentration (0.76 ± 0.16 mg/m^3), (3) moderate concentration (2.60 ± 0.19 mg/m^3), and (4) high concentration (9.78 ± 0.29 mg/m^3). The rats were exposed to graphene oxide for 6 h/day for 5 days, followed by recovery for 1, 3, and 21 days. No significant body or organ weight changes were noted after the short-term exposure or during the recovery period. Similarly, no significant systemic effects of toxicological importance were noted in the hematological assays, bronchoalveolar lavage fluid (BAL) inflammatory markers, BAL fluid cytokines, or blood biochemical assays following the graphene oxide exposure or during the post-exposure observation period. Moreover, no significant differences were observed in the BAL cell differentials, such as lymphocytes, macrophages, or polymorphonuclear cells. Graphene oxide-ingested alveolar macrophages as a spontaneous clearance reaction were observed in the lungs of all the concentration groups from post 1 day to post 21 days. Histopathological examination of the liver and kidneys did not reveal any significant test-article-relevant histopathological lesions. Importantly, similar to previously reported graphene inhalation data, this short-term nose-only

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inhalation study found only minimal or unnoticeable graphene oxide toxicity in the lungs and other organs.

Keywords

graphene oxide; short-term inhalation study(STIS); toxicology; nose-only inhalation exposure system

Introduction

Graphene is defined as a single layer of carbon atoms, with each atom bound to three neighbors in a honeycomb structure (ISO TS 80004–3, 2010). In the case of graphene oxide (GO), epoxide (1, 2 ether) and hydroxyl functional groups are covalently bonded on each side of the basal plane, while carboxyl groups are located at the edge sites (Balapanuru et al., 2010). This strong and light nanomaterial is expected to be used in many areas of industry, including biomedical applications, electronics, energy, and sensors, yet this also raises concerns about human exposure and hazards in environmental and occupational settings (Sanchez et al., 2012; Yang et al., 2015; Lee et al., 2016; Park et al., 2017). Occupational exposure to graphene nanomaterials can occur in air during the manufacture of graphene nanomaterials by oxidation and reduction processes, where the exposed forms can include individual graphene nanoplates, aggregates or agglomerates (Lee et al, 2016). Graphene oxide (200 – 500 nm lateral size) has already been found to induce cytotoxicity and genotoxicity in human lung fibroblasts in a dose-dependent manner (1 – 100 μm/ml), where oxidative stress and the surface charge of graphene oxide have been shown to mediate the toxicity (Wang et al., 2013). On the cellular level, graphene oxide (160 – 780 nm) did not enter A549 cells, yet caused oxidative stress dose-dependently and only induced a slight loss of cell viability at high concentrations, suggesting that graphene oxide is a relatively safe material (Chang et al., 2011). Moreover, in open field tests and functional observational battery tests, graphene oxide injections into rat tail veins for 7 days at concentrations of 2.5, 5, and 10 mg/kg body weight did not produce behavioral changes, yet caused inflammation of the lungs, liver, and spleen at a high concentration (10 mg/kg). The graphene oxide injections also reduced the cholesterol, high density lipoprotein (HDL), and low-density lipoprotein levels (Li et al., 2016). Another graphene oxide acute inhalation toxicity study also showed that acute inhalation exposure to graphene oxide induced minimal toxic responses in the lungs of male Sprague-Dawley rats at concentrations of 0.46 and 3.76 mg/m^3 without increasing the inflammatory markers (Han et al., 2015). Notwithstanding, the surface reactivity, size, and dispersion status of graphene nanomaterials all play an important role in the induction of toxicity and biodistribution of graphene nanomaterials. Plus, oxidative stress and the induction of an inflammatory response are also important for the induction of toxicity related to the biodistribution of graphene nanomaterials (Ema et al., 2017). Therefore, despite several acute inhalation toxicity studies of graphene oxide, there has been no repeated inhalation toxicity study of graphene oxide, which is more relevant for assessing the hazards from repeated exposure to graphene nanomaterials in the workplace. Accordingly, this study gathered the necessary data for a safety evaluation of graphene oxide, including its pulmonary toxicity, systemic toxicity via hematology, blood

biochemistry, and histopathology in the major organs following 5 days of exposure and after various post-exposure periods.

2. Materials and Methods

2.1 Characterization of graphene oxide

The graphene oxide nanopowder (GO-A200, IGH20160414; Thickness 1~2 atom layer) used in the experiments was provided by Grapheneall Co. (Gwinsean-gu, Suwon-si, Gyeonggi-do, South Korea). The physicochemical properties of the graphene oxide nanopowder were characterized, including its elemental contents, lateral size, thickness, D/G ratio, crystallinity and conductivity. The elemental contents were analyzed using a thermogravimetric analysis (TG/DTA 7300, Seiko Inc., Chiba, Japan) and inductive coupled plasma mass spectrometer (ICP-MS, Agilent Technologies 7300, Santa Clara, CA), the D/G ratio determined by Raman spectroscopy (WITec alpha 300, Ulm, Germany), the structural analysis conducted using a Rigaku Ultima IV X-ray diffractometer (Tokyo, Japan), the zeta-potential measured using a Malvern ZS90, He-Ne 633 laser (UK), the viscosity measured using a viscometer (PCE RVI 6, Southampton Hampshire, UK), the surface area measured using a BELSOPR-min II (MicrotracBEL, Osaka, Japan) based on the Brunauer-Emmett-Teller Method, and the thickness of the graphene layers analyzed using atomic force microscopy (AFM, Park System NX 10, Seoul, Korea). The graphene oxide aerosols were collected on a TEM grid (copper grid, Formvar/Carbon 200 mesh, TEDpella, CA) and analyzed using a field emission-transmission electron microscope (FE-TEM, JEM2100F, JEOL, Tokyo, Japan) equipped with an EDX (EDX, TM200, Oxford Instruments plc, Oxfordshire, UK) at an acceleration voltage of 200 k V (NIOSH 1994).

2.2 Aerosol generation

Male Sprague-Dawley (SD) rats were exposed to the graphene oxide nanopowder for 5 days using a nose-only exposure system (HCT, Icheon, Korea). The graphene oxide nanopowder was generated using an atomizer (AG-01, HCT, Icheon, Korea) (Table S1) with purified air as the carrier gas. Fresh air was used for the control, while various water suspensions were used to generate different aerosols: 0.04 mg/ml for the low concentration, 0.19 mg/ml for the moderate concentration, and 0.77 mg/ml for the high concentration. The air flow was maintained at 30 liters per minute (L/min) using a mass flow controller (MFX, FX-7810CD-4V, AERA, Tokyo, Japan), and the flow rate to each nose port was 1 L/min. The AC power supply was maintained at 99.56 ± 0.07 V (mean ± SE). The target concentrations of graphene oxide nanopowder were 0.625, 2.5, and 10 mg/m^3 for the low, moderate, and high concentration, respectively. The mass median aerodynamic diameter (MMAD) was measured using a MOUDI 125NR (cascade impactor, MSP Co, Shoreview, MN) at a flow rate of 10 L/min. Each stage used an aluminum foil filter coated with grease to minimize any particle bounce. The aerosol mass collected on the filters was determined as the difference between the post- and pre- weights of the filters. The geometric standard deviation (GSD) of the distribution was derived from the cumulative mass distribution for the filters.

2.3 Monitoring of graphene oxide aerosol in inhalation chamber

The particle size distribution was measured using a dust monitor (Model 1.1.09, Grimm Technologies Inc. Douglasville, GA) and scanning nanoparticle sizer (SMPS, HCT Co., Ltd., Icheon, Korea). The mass concentration of graphene oxide was determined gravimetrically (as post-weight minus pre-weight) by sampling on a PVC filter (polyvinyl chloride, size: 37mm and pore size 5.0 μm) at a flow rate of 1.0 L/min.

2.4 Elemental carbon analysis

To quantify the elemental carbon (EC) contents in the graphene oxide aerosols, quartz filters (37 mm diameter quartz fiber filters, SKC Inc., Eighty-Four, PA, USA) were also used to sample the total suspended particulate (TSP) and analyze the EC concentration. The quartz filters were subsequently analyzed to determine the airborne mass concentration of EC. The filters were analyzed according to NIOSH Manual of Analytical Method (NMAM) Method 5040 (NIOSH, 2003), which is currently recommended by NIOSH to assess exposure to CNTs and carbon nanofibers (CNF) (NIOSH, 2013). An organic carbon (OC) analysis is also routinely used to characterize carbonaceous nanomaterials as well as carbonaceous impurities in engineered nanomaterials (ENM). In this study, the reporting limit of quantitation (LOQ) for EC, organic carbon, and total carbon was 2 μg, 2 μg, and 4 μg/filter, respectively, and the limit of detection (LOD) was 0.6 μg/filter for each analyte category.

2.5 Animals and conditions

The six-week-old male specific-pathogen free SD rats were purchased from OrientBio (Seongnam, Korea) and acclimated for two-weeks before initiating the inhalation exposure. During the acclimation and inhalation exposure, the rats were kept in a controlled temperature (22 ± 0.83℃) and humid (47 ± 0.69 %) environment with 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 6 h/day. The rats were randomly divided into four groups: control (n=15), low-concentration (n=15), moderate-concentration (n=15), and high-concentration (n=15) groups. The low-, moderate-, and high-concentration groups were exposed to the graphene oxide nanopowder for 6 h/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, 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 oxide exposure, the rats were allowed to recover for 1, 3, and 21 days (n=5 per treatment group for each time period) to investigate clearance. All the animal experiments were approved by the Hanyang University Institutional Animal Care and Use Committee.

2.6 Organ weights, gross pathology, and histopathology

After collecting blood samples, the rats were euthanized using the anesthesiant Entobar®, followed by careful removal of the testes, heart, thymus, trachea, lungs, kidneys, spleen, liver, and brain. The organs were examined for any gross lesions, and then weighed and fixed in a 10 % formalin solution containing neutral phosphate-buffered saline (PBS). For

the histopathological evaluation, the testes were fixed in a Bouin solution, at killing, while the left lungs were fixed in a 10 % formalin solution (BBC Biochemical, Washington, DC) containing neutral phosphate-buffered saline under 25 cm of water pressure. After fixing the organs in 10% natural PBS for one week, they were then embedded in paraffin and stained with hematoxylin and eosin (BBC biochemical, Washington, DC). All the animal organs were examined using light microscopy. The left lungs were sectioned into three and examined.

2.7 Hematology and blood biochemistry

Following an intraperitoneal injection of the anesthesiant Entobar® (1 ml/kg) and prior to euthanasia, blood samples were drawn from the abdominal aorta into EDTA tubes for a hematological assay and separation tubes to determine the blood biochemistry. The blood was analyzed using a blood analyzer (Hitachi 7108, Hitachi, Tokyo, Japan), while the hematology was analyzed using a blood cell counter (Hemavet 0950, CDC Tech., Dayton, OH). The blood coagulation was analyzed using blood coagulation equipment (ACL700, Instrumentation Laboratory, Bedford, MA).

2.8 Bronchoalveolar lavage (BAL) cell analysis and measurement of inflammatory markers and cytokines in BAL fluid

At sacrifice, the right lungs were injected 4 times with 3 ml aliquots of warm calcium- and magnesium-free phosphate buffered saline (PBS) (pH 7.4). The BAL fluids were then centrifuged for 7 min at 500 × g, and the BAL cells collected and re-suspended in 1 ml of PBS for evaluation. The total cell number was determined using a hemocytometer. The cells were first smeared and then stained with Wright Giemsa Sure Stain to allow counts of the total number of cells, macrophages, polymorphonuclear cells (PMNs), and lymphocytes. Two hundred cells were evaluated for the cell differentiation. Furthermore, the BAL samples were also analyzed using a blood biochemical analyzer (Hitachi 7108, Hitachi, Japan) to determine the levels of lactate dehydrogenase (LDH), micro-albumin (mALB), micro-total protein (mTP), and blood urea nitrogen (BUN). The levels of inflammatory cytokines (TNF-α, IL-1β) in the BAL fluid were measured using a Quantikine Rat IL-1β/IL-1F2 Immunoassay (R&D Systems, Inc., Minneapolis, MN) and Quantikine Rat TNF-α Immunoassay (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions (principle: Sandwich enzyme immunoassay)

2.9 Lung deposition and dose calculation

The daily lung burden per rat was estimated for 6 h of continuous exposure, 1 minute ventilation of 0.19 L/min (Whalan et al., 2006), and the following aerosol properties (see Results section): 203 nm particle MMAD, 2.01 GSD, 15.36 % lung deposition efficiency based on MPPD (2002) (Multiple-path particle Dosimetry) Model v.2.0, and graphene oxide aerosol concentrations of 0.76, 2.60, and 9.78 mg/m^3 for the low, moderate, and high concentration, respectively. The following calculations were made:

> Daily deposited dose (mg/day) = average graphene oxide aerosol concentration (mg/m^3) × minute volume (L/min = 0.06m^3/h) × exposure duration (h/day) × deposition efficiency (1)

Deposition with low dose = 0.76 × (0.19 × 0.06) × 6 × 0.154 = 0.008 mg/day

Deposition with moderate dose = 2.60 × (0.19 × 0.06) × 6 × 0.154 = 0.027 mg/day

Deposition with high dose = 9.78 × (0.19 × 0.06) × 6 × 0.154 = 0.103 mg/day

Cumulative dose (mg)/animal = daily deposited dose (mg/day) × number of days (5)

For low exposure: 0.008 × 5 = 0.04 mg

For moderate exposure: 0.027 × 5 = 0.135 mg

For high exposure: 0.103 × 5 = 0.515 mg

2.10 Statistical analysis

The statistical analysis of the outcome parameters was performed using SPSS version 19 (SPSS Inc., Chicago, IL). The statistical evaluation was performed using an analysis of variance (ANOVA) following multiple comparison tests using the Dunnett T3 method. The level of statistical significance was set at $p < 0.05$, $p < 0.01$.

3. Results

3.1 Characteristics of graphene oxide nanopowder

The physicochemical characteristics of the graphene oxide nanopowder are shown in Table 1 and Figure 1. The carbon and oxygen contents were 42–45 % and 35–40 %, respectively, based on a thermogravimetric analysis (TGA), while the impurities were manganese <0.001% and sulfur <2.0% based on inductively coupled plasma-optical emission spectrometry (ICP-OES). The thickness of the graphene oxide was approximately 1 nm with 1–2 atom layers, and the lateral size ranged from 0.5 to 5 μm. A field emission TEM analysis of the graphene oxide nanopowder following aerosol generation revealed a stacked platelet structure with various thicknesses, ranging from 5.94 to 209.1 nm (Figure 2). Plus, a TEM-EDS analysis indicated the presence of two elements (i.e. C and O) (Figure 2). Table 2 shows the atomic percentages of the major graphene oxide components based on an EDS analysis: carbon (72.69 %), oxygen (27.31 %).

3.2 Monitoring chamber and graphene oxide distribution

The target mass concentrations of graphene oxide nanopowder were 0.625 mg/m^3, 2.5 mg/m^3, and 10 mg/m^3 for the low, moderate, and high concentration, respectively. The mass concentrations delivered in the low, moderate, and high concentration chambers were 0.76 ± 0.10, 2.60 ± 0.19, and 9.78 ± 0.29 mg/m^3, respectively (Table 3). The number concentrations in the chambers, measured using an optical particle counter (OPC), were 3.25 × 10^3 ± 1.18 × 10^2, 6.30 × 10^3 ± 2.90 × 10^2, and 9.97 × 10^3 ± 1.68 × 10^3 particle/cm^3 for the low, moderate, and high concentration, respectively (Table 3). The particle number concentrations were maintained as shown in Figure 3. The particle size distribution in the chambers was measured using a scanning mobility particle sizer (SMPS) (range 5.94 nm – 224.7 nm) and OPC (range 265 nm - 34 μm). The SMPS showed a peak at 22.5 nm, 25 nm, and 25.9 nm for the low, moderate, and high concentration, respectively (Figure 4A), while the OPC showed a peak at 265 nm, 365 nm, and 375 nm, respectively (Figure 4B). The mass

median aerodynamic diameter (MMAD), measured using a 13-stage MOUDI 125NR cascade impactor, was 203 nm with a GSD of 2.01 (Figure 5).

3.3 Elemental carbon analysis

The total EC was 0.18 ± 0.20 mg/cm^2, 1.56 ± 0.54 mg/cm^2, 3.34 ± 0.71 mg/cm^2, and 7.25 ± 1.14 mg/cm^2 for the control-, low-, moderate-, and high-concentration chambers, respectively (Table 4).

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

No significant gross effects were observed during the exposure. Also, no significant body weight losses or food consumption changes were observed during the exposure and recovery periods (Table S2 and 3). However, the right lung weights were significantly higher for the high concentration at post-1day ($P < 0.05$), suggesting further verification of inflammation using histopathology, plus BAL cell and BAL fluid analyses (Table S4)

3.5 Histopathology

While the number of alveolar macrophages with ingested graphene oxide increased in a concentration-dependent manner (Figure 6), a gradual clearance of graphene oxide was observed during the 21-day post-exposure period. Notwithstanding, some macrophages with ingested graphene still persisted at day 21 of the post-exposure period (Figure 6, red arrows). No significant histopathological observations were noted in the peripheral airway epithelia, interstitial tissues, alveolar space, or vasculature in the liver and kidneys. Light microscopical observation revealed no distinct evidence of movement of the graphene-oxide-ingested macrophages to the lymph nodes adjacent to the bronchi. Moreover, there was no significant histopathological response from the lung parenchyma, and a solely adaptive response of lung macrophage clearance was noted even with low magnification (Figure 7). The histopathological examination of the liver and kidneys did not reveal any significant test-article-induced lesions.

3.6 Measurement of inflammatory markers and cytokines

The differential cell count in the BAL did not reveal any significant changes in the total number of cells, macrophages, lymphocytes, or PMN (Table 5). When comparing the BAL inflammatory biomarkers with the control group, a significant increase in mTP was observed at day 3 post exposure for the low-concentration group. Again, when compared with the control group, all the exposed groups showed a consistent significant decrease in mALB at each post-exposure time point, yet no significant change in BUN or LDH. (Table 6). Furthermore, the BAL fluid showed no significant changes in cytokines IL-1β or TNF-α during the post-exposure period (Table 6).

3.7 Effect on blood coagulation

When compared with the control group, none of the exposed groups shown any change in the PT and APTT blood coagulation markers during the 21-day post-exposure period (Table 7).

3.8 Hematology and Blood biochemistry

The mean corpuscular hemoglobin concentration (MCHC) showed a significant decrease ($P < 0.01$) in the low-concentration group at post 1 day (Table S7). Moreover, the MCHC showed a decrease ($P < 0.05$) in the moderate- and high-concentration groups at post 3 days, yet an increase ($P < 0.05$) in the high-concentration group at post 21 days (Table S8–9). The mean platelet volume (MPV) showed an increase ($P < 0.01$) in the low-concentration group at post 1 day (Table S7). The percent of unstained cells (LUC) also increased ($P < 0.01$) in the low-concentration group at post 1 day and increased ($P < 0.05$) in the low- and moderate-concentration groups at post 3 days (Table S7–8). The absolute count of large unstained cells (abs luc) increased ($P < 0.05$) in the moderate-concentration group at post 3 days (Table S8). However, while LUC exhibited a consistent trend of significant increases across all the post-exposure time points, these changes were all within the normal range of the numerical control values for this outcome.

The moderate-concentration group showed a lower cholesterol (CHO) level when compared with the low-concentration group ($p < 0.05$) (Table S10). The level of creatine (CRE) also decrease ($P < 0.05 – 0.01$) in the moderate- and high-concentration groups at post 1 day (Table S10). The inorganic phosphate (IP) decreased significantly ($P < 0.05 – 0.01$) in all the exposed groups at post 1 day, and decreased ($P < 0.05$) in the moderate- and high-concentration groups at post 3 days (Table S10–11). The level of lactate dehydrogenase (LDH) decreased ($P < 0.05$) in the high-concentration group at post 1 day and decreased ($P < 0.01$) in the moderate- and high-concentration groups at post 3 days (Table S10–11). The high-concentration group also showed a lower level of LDH than the low-concentration group ($p < 0.05$), (Table S10). The level of magnesium(MG) decreased ($P < 0.01$) in the moderate- and high-concentration groups at post 1 day and decreased ($P < 0.01$) in the low-, moderate-, and high-concentration groups at post 3 days. The moderate- and high-concentration groups also showed a lower level of MG than the low-concentration group ($P < 0.01$) (Table S 10–12). The level of triglyceride (TG) increased ($P < 0.01$) in the low-concentration group at post 1 day, and increased ($P < 0.01$) in the high-concentration group at post 3 days (Table S10–11). Yet, while the TG level showed consistent significant increases across the post-exposure time points, these changes were within the normal range of the numerical control values. The level of glutamic oxidative transaminase (GOT) decreased ($P < 0.01$) in the low-concentration group at post 1 day and increased ($P < 0.01$) in the high-concentration group at post 3 days (Table S11). The high-concentration group also showed a lower GOT when compared with the low-concentration group ($p < 0.05$) (Table S10). The level of creatine kinase (CK) decreased ($P < 0.01$) in the moderate- and high-concentration groups at post 3 days (Table S11). The amount of sodium (Na) decreased ($P < 0.05$) in the low-concentration group at post 3 days (Table S11). The levels of albumin (ALB) and alkaline phosphatase (ALP) decreased ($P < 0.05$) in the moderate- and low-concentration groups, respectively, at post 21 days (Table S12). The level of glucose (GLU) increased ($P < 0.01$) in the moderate- and high-concentration groups at post 21 days (Table S12). Yet, while consistent significant increases in IP, GOT, LDH, MG, CK were observed at all the post-exposure time points, these changes were all within the normal range of the numerical control values (Table S10–11). Consequently, no significant effects of

toxicological importance were noted in the hematological kidney and liver functions after graphene oxide exposure.

4. Discussion

The terms “safe innovation” and “safe-by-design” are currently widely used in the field of nanotechnology to advocate safety considerations at an early stage of the innovation process of nanomaterials and nano-enabled products (Park et al., 2017). Indeed, graphene nanomaterials are a good example of safe innovations or safe-by-design. Due to strong interest in the commercial development of graphene-relevant nanomaterials and the increasing production trend, evaluating the risks involved is very important before manufacturing starts. A recent review of graphene-based nanomaterial toxicity studies in laboratory animals indicated potential behavioral, reproductive, and developmental toxicities and genotoxicities (Ema et al., 2017).

While the acute inhalation toxicity of graphene oxide has been reported to be low (Han et al, 2015), the repeated inhalation toxicity of graphene oxide has not yet been studied. Therefore, the current study, based on the short-term inhalation study by Ma-hock et al (2009), represents an initial step for hazard assessment and range finding for future subacute and sub-chronic studies. As a result, the current short-term inhalation study found no significant systemic toxic effects of graphene oxide based on hematological and biochemical analyses following exposure to graphene oxide and during a post-exposure observation period. The results also showed a similar tendency to those of a previous subacute graphene exposure study (Kim et al., 2016). Histopathological examination of the liver and kidneys did not reveal any significant test-article-relevant histopathological lesions. Moreover, no significant differences were observed in the bronchoalveolar lavage cell differentials, such as the lymphocytes, macrophages, and PMNs. Evaluation of the BAL fluid cytokines (i.e. IL-1β, TNF-α) also showed no significant concentration-dependent changes during the post-exposure period (Table 6) A spontaneous clearance reaction of graphene-oxide-ingested alveolar macrophages was observed in the lungs from all the concentration groups throughout the 21-day post-exposure period. Table 8 presents a comparison of the toxicity results for graphene and graphene oxide in various short-term and subacute graphene inhalation studies. The short-term graphene inhalation study by Shin et al., 2015 and subacute graphene inhalation study by Kim et al., 2016 reported nearly identical toxicity results to the acute graphene oxide inhalation study by Han et al., 2015 and current short-term graphene oxide inhalation study. However, another graphene nanoplate pharyngeal aspiration study using < 2, 5, and 20 μm lateral sizes found increased inflammatory markers in the BAL fluids when using graphite nanoplates larger than 5 μm (Roberts et al., 2016). Such different results may have been due to the lateral size distribution of graphene and graphene oxide. When the lateral size was less than 5 μm, graphene- and graphene oxide-ingested alveolar macrophages showed similar results with minimum toxicity. However, when the lateral size was larger than 5 μm, graphene induced an inflammatory response in the BAL fluid post-exposure (Ma-hock et al., 2013). In the studies by Shin et al (2015) and Ma-hock et al (2013), animals were exposed to graphene nanoplates via inhalation at a maximum of 3 $\mu g/m^3$ and 10 $\mu g/m^3$, respectively, and allowed to recover for 21 days. While the graphene nanoplate aerosols in each study had a similar MMAD, the inflammatory

responses were determined by the different lateral sizes of the graphene nanoplates. Moreover, different delivery methods may also affect the inflammatory response. Intratracheal instillation (Shinwald et al., 2012) or pharyngeal aspiration techniques for delivery to the lungs of rats or mice can result in more aggregation of nanomaterials and generally do not accurately reproduce the deposition patterns attained with inhalation exposure to dry aerosolized or nebulized suspensions of nanomaterials. The main difference is that bolus exposure (instillation of aspiration) represents a non-physiological mode of delivering a liquid-suspended material within a fraction of a second at a very high dose rate, while physiological inhalation deposits aerosolized materials over an extended time period (days, weeks, or months at a low dose rate) (Oberdorster et al., 2015). Therefore, the inflammatory response induced after the intratracheal instillation of graphene oxide and reduced graphene oxide in mice with an increased acute phase response together with serum amyloid A (Bengston et al., 2017) was not observed in the current inhalation study, which showed no significant inflammatory response.

A subchronic graphene oxide nanoplate inhalation study was also completed based on the authors' short-term inhalation study, and the results showed macrophage-ingested particles in moderate and high concentrations as a spontaneous reaction for clearance. Hence, the suggested NOAEL for the subchronic inhalation study was 3.02 mg/m^3 and no target organs were identified (An et al., 2017).

Recently revised OECD test guidelines for subacute and subchronic inhalation toxicity tests now require a lung burden analysis to obtain information on pulmonary deposition and particle retention in the lungs after terminating exposure and at post-exposure observation intervals (OECD 2017a; 2017b). In the current study, elemental carbon (EC) measurements of lung tissue from post 1 day to 21 days were used to provide graphene oxide clearance information, however, there was no available technology for analyzing the EC from the lung tissue. Such technology has since been developed by the current authors, and will be used in our future carbon nanomaterial inhalation studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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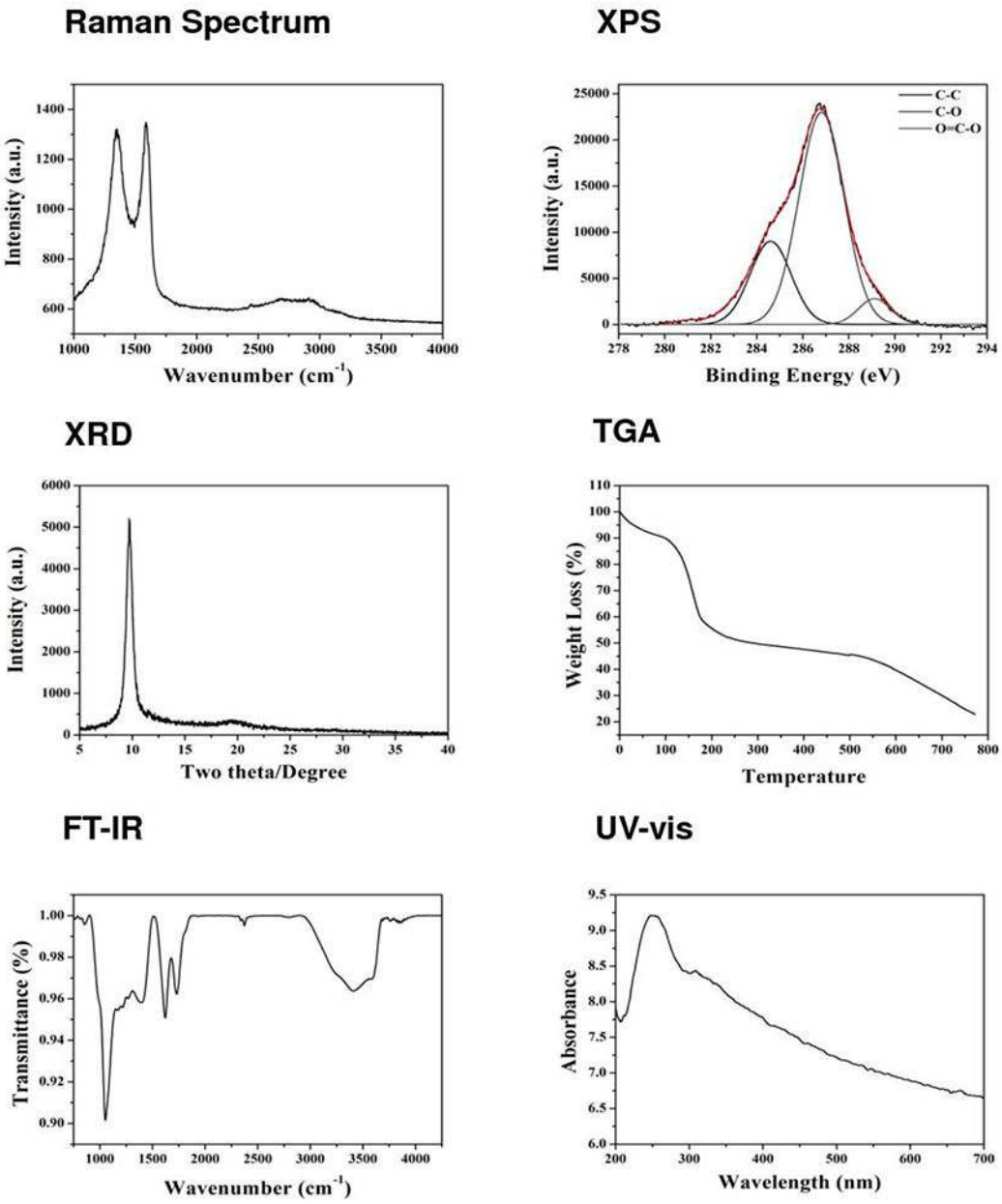

Figure 1.

Graphene oxide physicochemical characteristics. XPS, X-ray photoelectron spectroscopy; XRD, X-Ray Diffraction; TGA, Thermogravimetric Analysis; FT-IR, Fourier-transform infrared spectroscopy; UV-vis, Ultraviolet–visible spectroscopy

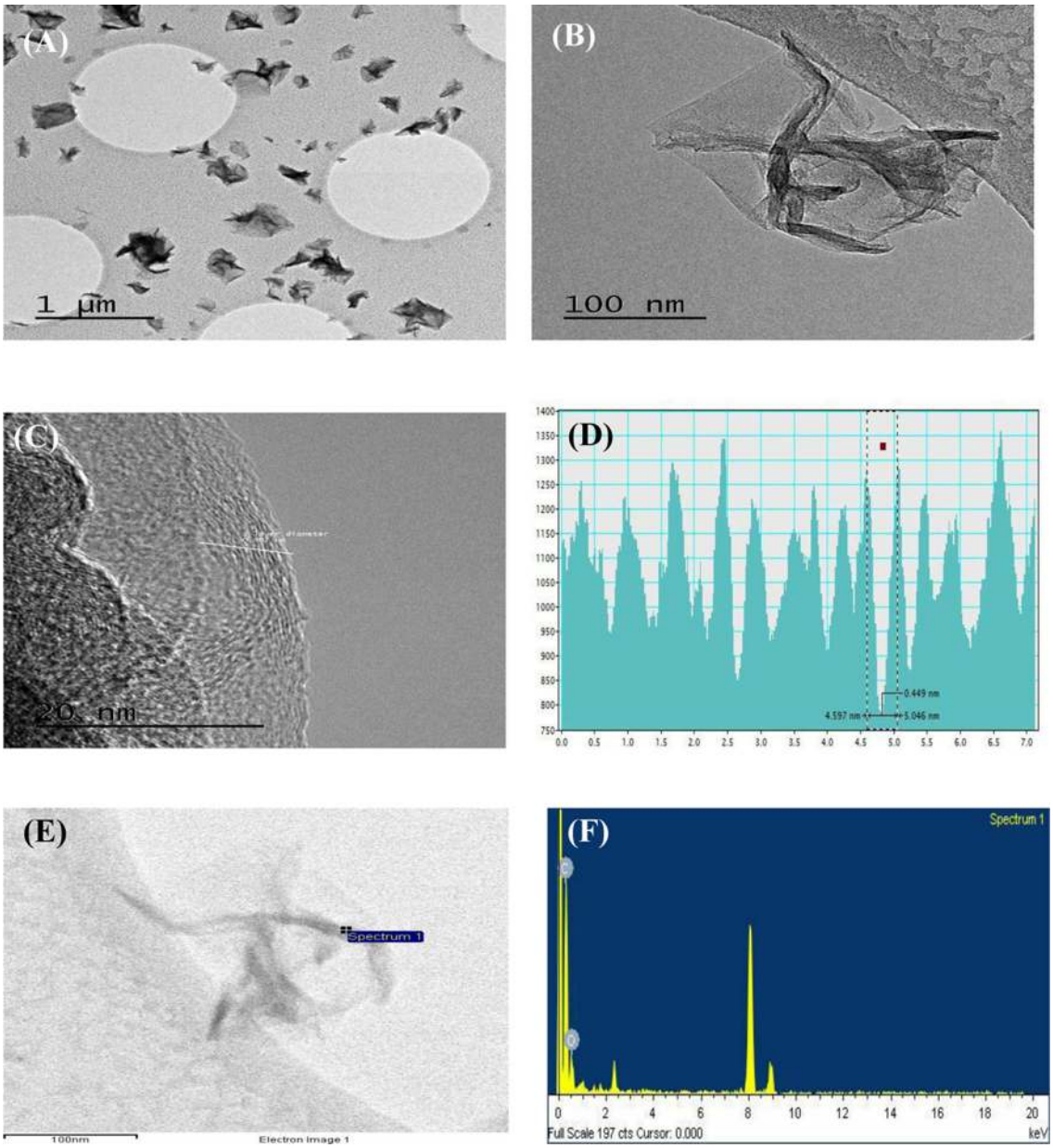

Figure 2.

Analysis of graphene oxide using FE-TEM (A-D) (Field emission-transmission electron microscopy) (×100,000). (E-F) EDS-spectrometer (energy dispersive spectroscopy).

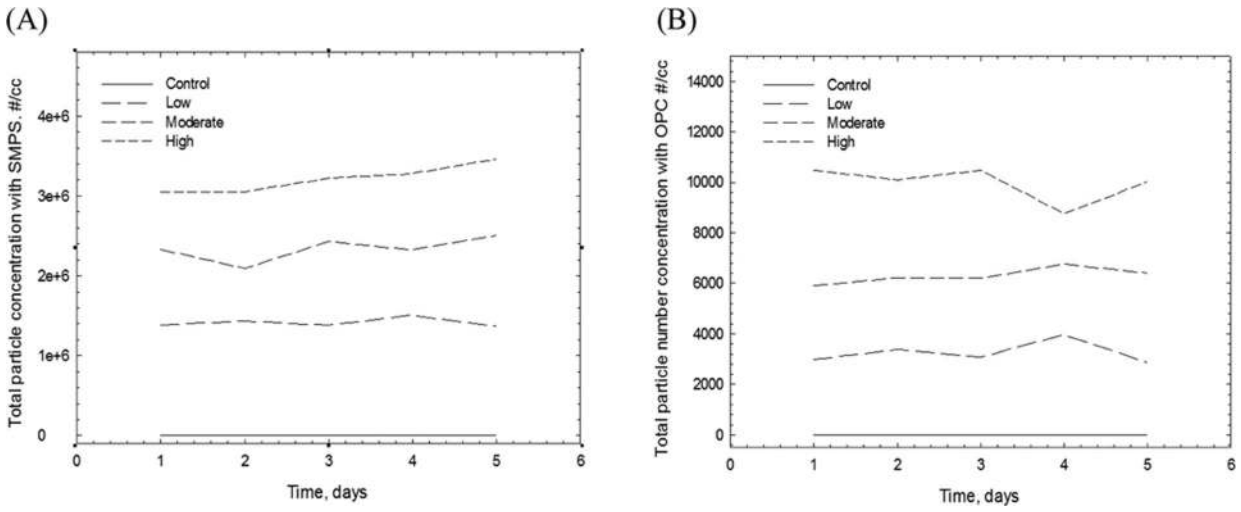

Figure 3.

Particle number concentrations in graphene oxide aerosols inside inhalation chambers during 5-day exposure measured using scanning mobility particle sizer (SMPS) (A) and optical particle counter (OPC) (B)

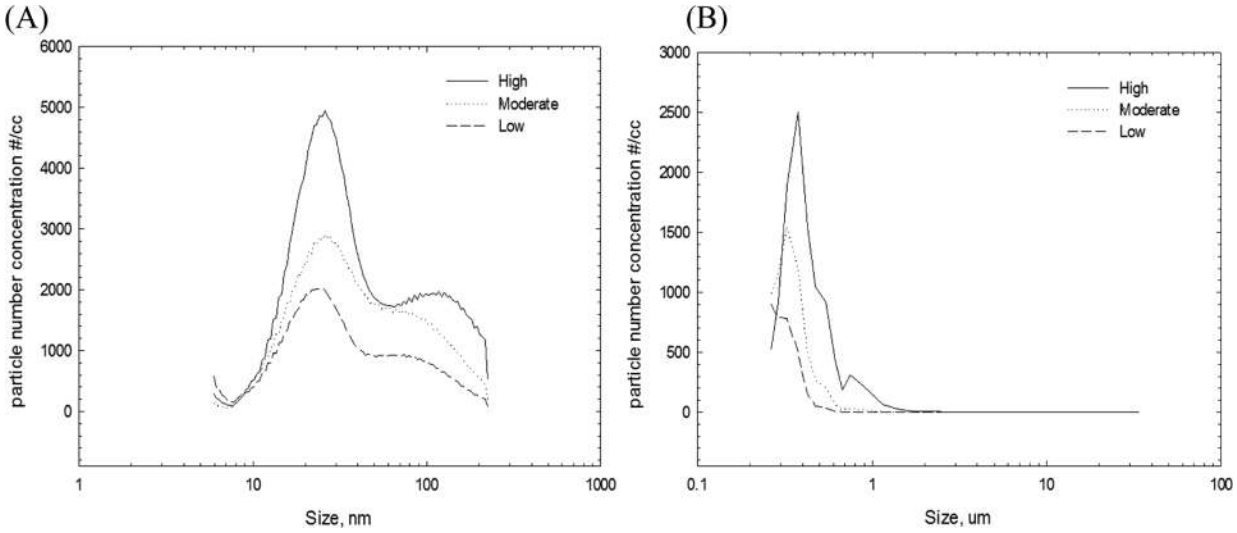

Figure 4.

Particle size distribution of graphene oxide aerosols inside inhalation chambers measured using SMPS (A) and OPC (B). Distributions were bimodal, with peak maxima at 20–30 nm (SMPS) and 300–400 nm (OPC), as shown in Table 3.

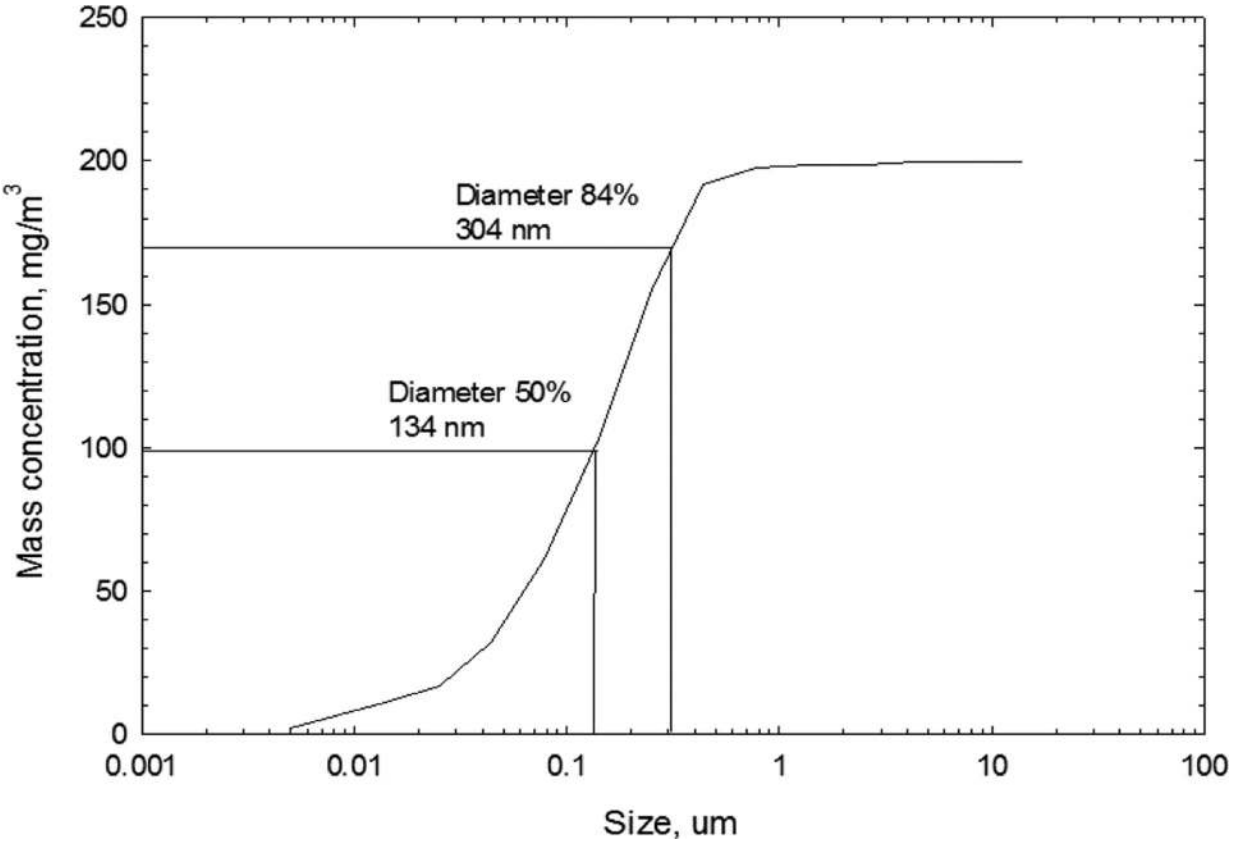

Figure 5.

Mass median aerodynamic diameter (MMAD) (203 nm) and geometric standard deviation (GSD) (2.01) of aerosolized graphene oxide measured using MOUDI

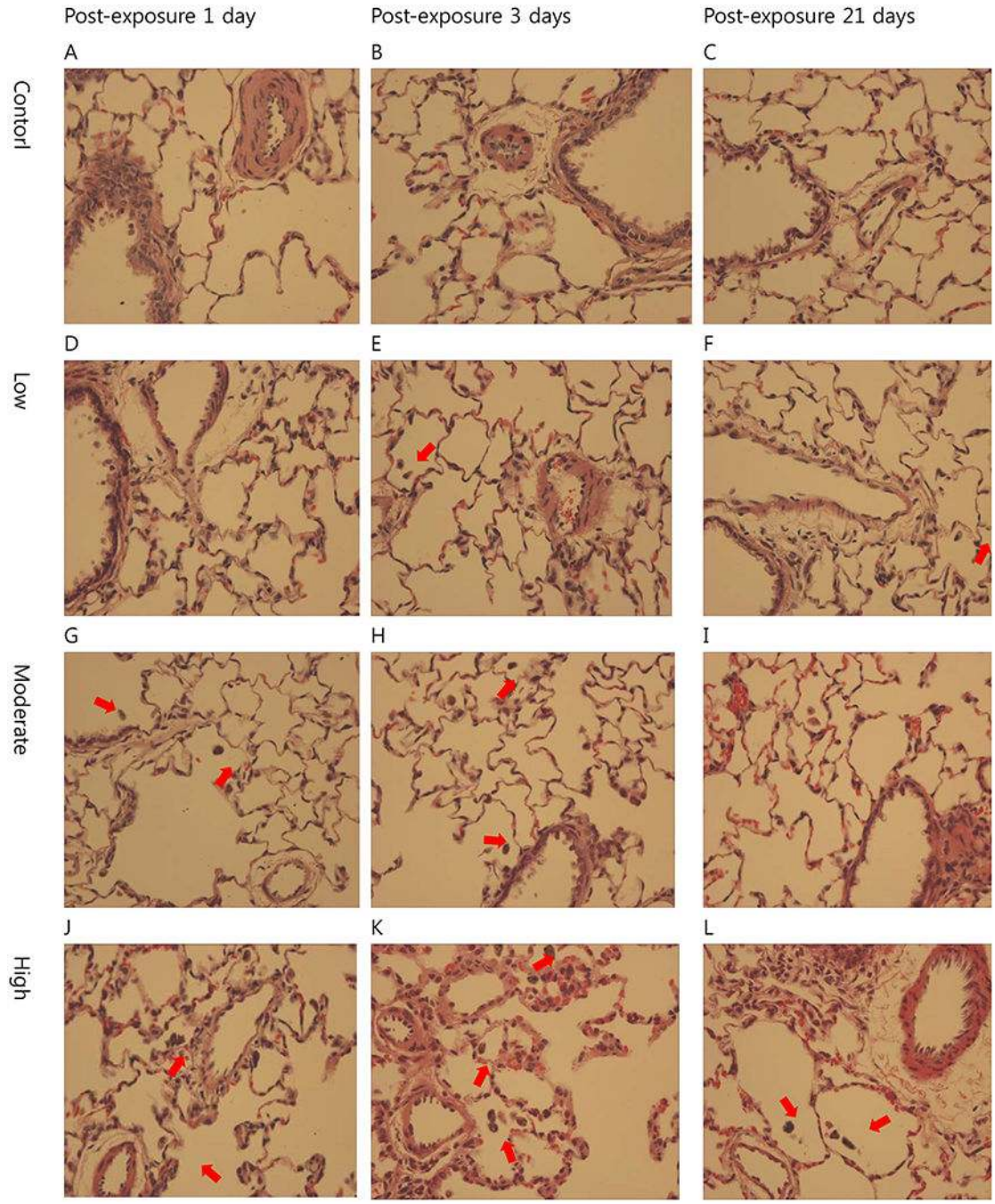

Figure 6.

Lung histopathology at post 1, 3, and 21 days. Panels are organized as post exposure day vs concentration. Magnification 400x. None of micrographs show any inflammation in bronchiols or perivascular regions. There were no fibrotic cell proliferations in interstitial tissues. Macrophages with ingested graphene oxide were noted with concentration-dependency. No granulomatous appearance was observed throughout post-exposure period at various concentrations. Red arrows indicate macrophages with ingested graphene oxide

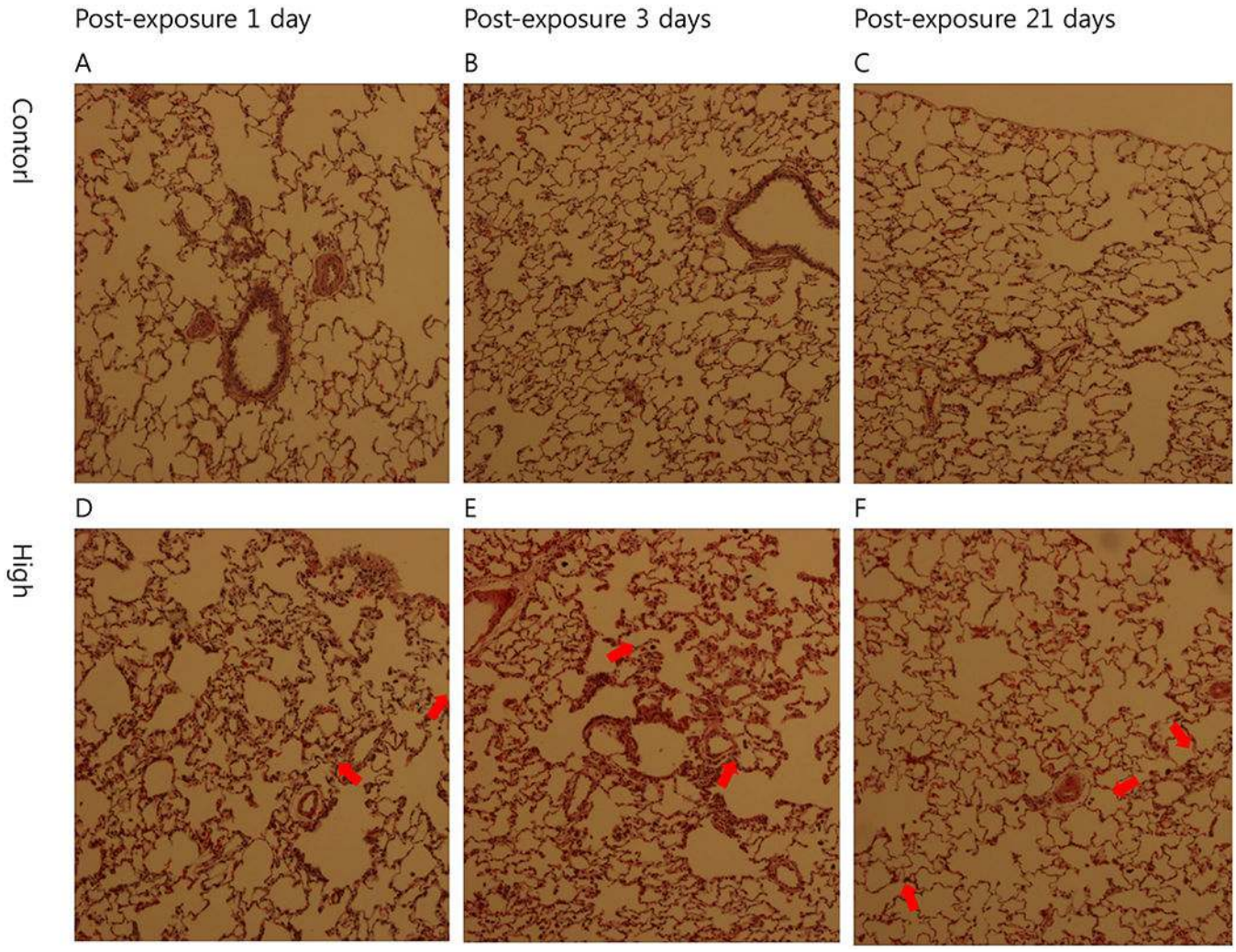

Figure 7.

Lung histopathology at post 1, 3, and 21 days. Panels show micrographs of control and high concentration with low magnification (100x). None of micrographs show any inflammation in bronchiols or perivascular regions or lung parenchyma. Red arrows indicate macrophages with ingested graphene oxide.

Table 1.

Graphene oxide physicochemical information

Specification	Unit of measure	Value	Method of analysis
Total graphene oxide content	Wt %	93.44 ±1.20	TGA
Carbon content	%	42–45	TGA
Oxygen content	%	35–40	TGA
Impurities: -Manganese -Sulfur	%	< 0.001 < 2.0%	ICP-OES
Thickness	nm	1–2 atomic layer (~1nm > 98 %)	AFM, TEM
Lateral Size	μm	0.5 – 5	PSA, TEM, SEM
D/G ratio	-	1.01	Raman
XRD 2θvalue	-	9.7 (d'spacing = 0.91 nm)	XRD
Solution conductivity	mS/m	30 – 70	SCM
Density	g/cc	~ 1.7	DM
Zeta potential	mV	−40 mV, pH	ZPA
Viscosity		1.98 (g/L, 22°C)	NVM
Surface area	m^2/g	8.4645	BET

TGA, Thermogravimetric Analysis; ICP-OES, inductively coupled plasma-optical emission spectrometry; XRD, X-ray diffraction; AFM, atomic force microscopy; TEM, transmission electron microscopy; SEM, scanning electron microscope; PSA, particle size analyzer; ZPA, zeta potential analyzer; SCM, solution conductivity meter; DM, density meter; NVM, Nano-viscosity meter; BET Brunauer-Emmett-Teller Method

Table 2.

EDS analysis of graphene oxide

ELEMENT	WEIGHT (%)	ATOMIC (%)
C	66.64	72.69
O	33.36	27.31
Total		100.00

Table 3.

Real-time monitoring and mass concentration of graphene oxide nanopowder in chambers

DISTRIBUTION OF GRAPHENE OXIDE				
Group (mean ± SE)	**Control**	**Low**	**Moderate**	**High**
SMPS (particles/cm^3)	0	$1.42\times10^6 \pm 5.56\times10^4$	$2.34\times10^6 \pm 1.56\times10^5$	$3.21\times10^6 \pm 1.72\times10^5$
OPC (count/L)	0	$3.25\times10^3 \pm 1.18\times10^2$	$6.30\times10^3 \pm 2.90\times10^2$	$9.97\times10^3 \pm 1.68\times10^2$
Mass Concentration (mg/m^3)	0	0.76 ± 0.10	2.60 ± 0.19	9.78 ± 0.29

Data are presented as mean ± standard deviation

Table 4.

Elemental carbon concentration in generated graphene oxide in chambers (mean ± SD).

SUMMARY OF ELEMENTAL CARBON ANALYSIS				
Unit: mg/cm^2				
Group (mean ± SE)	**Control**	**Low**	**Moderate**	**High**
Total carbon	1.40 ± 0.92	3.50 ± 0.77	5.53 ± 0.86	9.66 ± 1.32
Elemental carbon	0.18 ± 0.20	1.56 ± 0.54	3.34 ± 0.71	7.25 ± 1.14
Organic carbon	1.22 ± 0.72	1.94 ± 0.26	2.18 ± 0.18	2.41 ± 0.20

Data are presented as mean ± standard deviation

Table 5.

Bronchoalveolar cell count at 1, 3, and 21 days post exposure

SUMMARY OF BRONCHOALVEOLAR LAVAGE ANALYSIS																
UNIT: ×10⁶/mL													SEX : MALE			
Group: x10⁶/ml (mean ± SE)	**Control**				**Low**				**Moderate**				**High**			
Post 1 day																
Total cell count	0.46	±	0.07	(5)	0.56	±	0.06	(5)	0.67	±	0.13	(5)	0.65	±	0.11	(5)
Macrophages	0.41	±	0.07	(5)	0.51	±	0.07	(5)	0.61	±	0.12	(5)	0.61	±	0.10	(5)
Lymphocytes	0.04	±	0.01	(5)	0.05	±	0.01	(5)	0.06	±	0.01	(5)	0.04	±	0.01	(5)
PMN	0.00	±	0.00	(5)	0.00	±	0.00	(5)	0.00	±	0.00	(5)	0.00	±	0.00	(5)
Post 3 days																
Total cell count	0.45	±	0.05	(5)	0.50	±	0.04	(5)	0.78	±	0.10	(5)	0.71	±	0.16	(5)
Macrophages	0.40	±	0.05	(5)	0.45	±	0.04	(5)	0.71	±	0.09	(5)	0.65	±	0.15	(5)
Lymphocytes	0.05	±	0.00	(5)	0.05	±	0.01	(5)	0.07	±	0.02	(5)	0.05	±	0.01	(5)
PMN	0.00	±	0.00	(5)	0.00	±	0.00	(5)	0.00	±	0.00	(5)	0.00	±	0.00	(5)
Post 21 days																
Total cell count	0.53	±	0.04	(5)	0.47	±	0.08	(5)	0.50	±	0.05	(5)	0.45	±	0.09	(5)
Macrophages	0.48	±	0.04	(5)	0.41	±	0.06	(5)	0.45	±	0.00	(5)	0.42	±	0.09	(5)
Lymphocytes	0.05	±	0.01	(5)	0.05	±	0.01	(5)	0.05	±	0.01	(5)	0.03	±	0.01	(5)
PMN	0.00	±	0.00	(5)	0.00	±	0.00	(5)	0.00	±	0.00	(5)	0.00	±	0.00	(5)

( ): number of animals

Table 6.

BALF chemistry and cytokine levels in BAL fluid from rats at 1, 3, and 21 days post exposure

Group: (mean ± SE)	Control	Low	Moderate	High
Post 1 day				
Serum BUN[1]	15.85 ± 0.56 (5)	14.60 ± 0.85 (5)	15.22 ± 0.84 (5)	13.23 ± 0.89 (5)
BALF BUN[2]	0.24 ± 0.02 (5)	0.25 ± 0.02 (5)	0.28 ± 0.01 (5)	0.23 ± 0.02 (5)
BALF LDH[3]	27.80 ± 2.08 (5)	26.20 ± 2.56 (5)	28.20 ± 4.31 (5)	25.60 ± 1.03 (5)
BALF mALB[4]	10.62 ± 1.00 (5)	9.31 ± 0.89 (5)	9.67 ± 1.17 (5)	9.41 ± 0.65 (5)
BALF mTP[5]	6.34 ± 0.35 (5)	6.08 ± 0.28 (5)	6.06 ± 0.45 (5)	6.20 ± 0.16 (5)
IL-1β[6]	0.46 ± 0.07 (5)	0.56 ±0.06 (5)	0.67 ± 0.13 (5)	0.47 ± 0.11 (5)
TNF-α[7]	0.41 ± 0.07 (5)	0.51 ± 0.07 (5)	0.61 ± 0.12 (5)	0.61 ± 0.10 (5)
Post 3 days				
Serum BUN[1]	15.98 ± 0.35 (5)	16.32 ± 1.02 (5)	16.01 ± 0.84 (5)	15.12 ± 0.56 (5)
BALF BUN[2]	0.27 ± 0.03 (5)	0.31 ± 0.02 (5)	0.26 ± 0.02 (5)	0.26 ± 0.03 (5)
BALF LDH[3]	26.60 ± 0.81 (5)	30.20 ± 1.83 (5)	28.00 ± 2.07 (5)	29.20 ± 4.22 (5)
BALF mALB[4]	9.65 ± 0.76 (5)	12.01 ± 1.12 (5)	7.43 ± 0.62 (5)	8.61 ± 0.49 (5)
BALF mTP[5]	6.02 ± 0.28 (5)	7.09 ± 0.26 (5) *	5.83 ± 0.20 (5)	6.10 ± 0.21 (5)
IL-1β[6]	0.45 ± 0.05 (5)	0.50 ± 0.04 (5)	0.78 ± 0.10 (5)	0.71 ± 0.16 (5)
TNF-α[7]	0.40 ± 0.05 (5)	0.45 ± 0.04 (5)	0.71 ± 0.09 (5)	0.65 ± 0.15 (5)
Post 21 days				
Serum BUN[1]	16.90 ± 0.59 (5)	15.45 ± 1.21 (5)	14.16 ± 0.70 (5)	15.25 ± 0.49 (5)
BALF BUN[2]	0.25 ± 0.02 (5)	0.28 ± 0.05 (5)	0.16 ± 0.02 (5)	0.20 ± 0.03 (5)
BALF LDH[3]	34.80 ± 2.71 (5)	31.60 ± 5.27 (5)	30.80 ± 2.27 (5)	23.80 ± 2.22 (5)
BALF mALB[4]	10.10 ± 1.11 (5)	17.89 ± 4.46 (5)	11.19 ± 0.65 (5)	11.54 ± 1.13 (5)
BALF mTP[5]	6.96 ± 0.26 (5)	9.68 ± 1.55 (5)	7.97 ± 0.37 (5)	7.86 ± 0.39 (5)
IL-1β[6]	0.53 ± 0.04 (5)	0.47 ± 0.08 (5)	0.50 ± 0.05 (5)	0.45 ± 0.09 (5)
TNF-α[7]	0.48 ± 0.04 (5)	0.41 ± 0.66 (5)	0.45 ± 0.00 (5)	0.42 ± 0.09 (5)

( ): number of animals;

[1] Serum blood urea nitrogen (mg/dL)

[2] Blood urea nitrogen (mg/dL);

[3] Lactate dehydrogenase (KU/L/DrDF);

[4] Micro albumin (mg/ml/UrDF);

[5] Micro total protein (mg/ml/UrDF),

[6] IL-1 β, Interleukin-1 beta (ng/mL/UrDF);

[7] TNF-α, Tumor necrosis factor-alpha (ng/mL/UrDF); UrDF, urinary dilution factor;

*, P<0.05 compared with control group

Table 7.

Blood coagulation analysis of male rats exposed to graphene oxide for 5 days followed by 1, 3, and 21 days of recovery.

SUMMARY OF BLOOD COAGULATION ANALYSIS																
													SEX: MALE			
Group (mean ± SE)	**Control**				**Low**				**Moderate**				**High**			
Post 1 day																
PT[1] (sec)	9.39	±	0.15	(5)	9.75	±	0.28	(5)	9.94	±	0.19	(5)	9.69	±	0.13	(5)
APTT[2*] (sec)	-	±	-	(5)	-	±	-	(5)	-	±	-	(5)	-	±	-	(5)
Post 3 days																
PT[1] (sec)	9.49	±	0.24	(5)	9.36	±	0.29	(5)	9.08	±	0.21	(5)	9.15	±	0.08	(5)
APTT[2] (sec)	12.70	±	0.51	(5)	13.62	±	0.75	(5)	14.48	±	1.33	(5)	14.42	±	0.79	(5)
Post 21 days																
PT[1] (sec)	9.59	±	0.28	(5)	9.61	±	0.18	(5)	9.63	±	0.48	(5)	8.85	±	0.16	(5)
APTT[2] (sec)	17.92	±	0.17	(5)	18.14	±	0.56	(5)	17.98	±	2.85	(5)	15.36	±	0.69	(5)

( ) number.

1. Prothrombin time;

2. Activated partial thromboplastin time

* No APTT analysis of sample errors during experiment post 1 day

Table 8.

Comparison of graphene and graphene oxide studies

	Graphene				Graphene oxide	
	Shin et al (2015)	Ma-Hock et al (2013)	Roberts et al (2016)	Kim et al (2016)	Han et al (2015)	Current
Exposure	Inhalation (STIS)	Inhalation (STIS)	Pharyngeal aspiration	Inhalation	Inhalation	Inhalation (STIS)
Duration	6 h/d, 5 d/week	6 h/d, 5 d/week	Once	6 h/d, 5 d/ week, 4 week	6 h/d, 1 day	6 h/d, 5 d/week
Post exposure	1, 7, 21 d	1, 3, 21 d	4 h, 1 d, 7 d, 1, 2 month	1, 28, 90 d	7, 14 d	1, 3, 21 d
Concentration	0.68, 3.86 mg/m^3	0.5, 2.5, 10 mg/m^3	4, 40 μg/mice	0.12, 0.47. . 88 mg/m^3	0.46, 3.76 mg/m^3	0.76, 2.60, 9.78 mg/m^3
MMAD	0.567 μm	< 0.4 μm	–	0.123 μm	–	0.134 μm
Test material dimensions	550 nm (lateral) 0.39–9.24 (thickness)	10 μm (lateral)	20, 5, < 2 μm (lateral)	2 μm (lateral)	150–250 nm (hydrodynamic size)	0.5–5 μm (lateral)
Results	Minimal Toxicity (graphene-ingested macrophages)	Increased inflammatory markers in BAL fluid from 2.5 mg/m^3 during post-exposure period	Increased lung inflammatory markers in BAL fluid at 40 μg/mouse	Minimal toxicity (graphene-ingested macrophages)	Minimal toxicity (graphene oxide-ingested macrophages)	Minimal toxicity (graphene oxide-ingested macrophages)

*STIS, short-term inhalation study; MMAD, mass median aerodynamic diameter