Graphene oxide nanosheets induced genotoxicity and pulmonary injury in mice

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**ABSTRACT**

Graphene and graphene-related materials have broadly applied in biomedical purposes due to their unique properties, thus safety evaluation of them is crucial. This study was performed to explore the genotoxic and pulmonary toxic potential of different doses of graphene oxide nanosheets’ (GOs) in mice. A total of 90 male mature mice were randomly divided into six groups of fifteen mice each per group, five groups were intraperitoneally injected by GO at doses of 10, 50, 100, 250 and 500 \(\mu\)g/kg b.w once weekly in addition to the control group that was injected intraperitoneally with 0.2 ml saline solution. Five animals from each group were euthanized after 7, 28 and 56 days post treatment. Evaluation of genotoxicity was performed through detection of chromosomal aberrations in bone marrow while assessment of lung injury was made by determination of DNA fragmentation in lung specimens using the alkali Comet assay, pulmonary oxidative markers estimation and finally histopathological investigations. Results revealed that GOs induced variable structural chromosomal aberrations (SCA) in bone marrow and DNA damage of lung cells that were time and dose dependent and represented by increase in %DNA in comet tail, tail moment and tail length and decrease in % head DNA in nuclei of lung of GOs-treated mice versus control groups in addition, GOs induced various changes in pulmonary oxidative stress parameters that were affected by dose and duration of treatment compared with the control as well as various pulmonary histopathological alterations were detected indicating lung injury. Conclusion: GO potentiate the induction of genotoxicity and pulmonary injury in mice in time and dose dependent manner.

1. Introduction

Carbon nanomaterials have various physical, chemical and biological characteristics enhancing their wide range use. Graphene is one of carbon based nanomaterials, a single-atom-thick, two-dimensional sheet of hexagonally arranged carbon atoms (Geim and Novoselov, 2007), Nobel Prize was awarded in 2010 for Andre K. Gem and Konstantin S. Novoselov for isolation and characterization of graphene from crystalline graphite (Novoselov et al., 2004). Graphene has unique characteristics as high surface area, extraordinary electrical and thermal conductivity, and strong mechanical strength (Geim and Novoselov, 2007; Li et al., 2008), encouraging their broad applications,

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**Abbreviations:** AS49, human lung adenocarcinoma; ANOVA, analysis of variance; b.wt, body weight; BEAS-2B, human bronchial epithelial cells; CAs, chromosomal aberrations; CAT, catalase; CNTs, carbon nanotubes; Cu, copper; DMSO, dimethylsulfoxide; DNA, deoxyribonucleic acid; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; EDTA, ethelyne diamine tetra acetic acid; G, graphite; gm, gram; GNFs, graphite nanofibers; GO, graphene oxide; GOs, graphene oxide nanosheets; GSH, glutathione (reduced glutathione); h, hour; HCl, hydrochloric acid; H&E, hematoxylin and eosin; H2O2, hydrogen peroxide; H2SO4, sulfuric acid; HR-TEM, high resolution transmission electron microscope; i.p, intraperitoneal; i.v., intravenous; IBN, International Berrian Nanotechnology; ICPDS, Joint Committee on Powder Diffraction Standards; K, kilogram; KMnO4, potassium permanganate; K, kilo volt; LaB6, lanthanum hexabromide; LSD, least significant difference; m, meter; mA, mili amber; MDA, malonialdehyde; Min, minute; ml, milliter; mM, millimole; MWCNTs, multi walled carbon nanotubes; NaCl, sodium chloride; NaNO3, sodium nitrate; NaOH, sodium hydroxide; NBT, nitroblue tetrazolium; NIH, National Institutes of Health; nm, nanometer; nmol, nanomolar; pH, negative logarithm of hydrogen ion concentration; ROS, reactive oxygen species; rpm, round per minute; SCA, structural chromosomal aberrations; SCE, single cell gel electrophoresis; SD, standard division; SEM, standard error of mean; SOD, superoxide dismutases; SPSS, statistical package for social sciences; SWCNTs, single walled carbon nanotubes; TBARS, thiobarbituric acid reactive substance; TEM, transmission electron microscopy; TIA, TEM imaging and analysis; TNA, total numerical aberrations; TRA, total structural aberrations; U, unit; V, vascular capillary; volt, voltage; XRD, X-ray diffraction; α, alpha; µl, microliter; µm, micrometer; µg, microgram; °C, degree centigrade; 2D, two-dimensional

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such as structural composites, conducting polymers (Stankovich et al., 2006), supercapacitors (Dikin et al., 2007), battery electrodes (Paek et al., 2009; Su et al., 2010), transport barriers (Bunch et al., 2008; Compton et al., 2010), printable inks (Wang et al., 2010), antibacterial papers and in biomedical technologies. Graphene oxide nanosheet is a heavily oxygenated graphene derivative with great stability in aqueous dispersion enhancing their use in biomedical researches and drug delivery (Liu et al., 2008; Guo and Mei, 2014), several researches have been conducted for evaluation of graphene and its derivatives in animal models via different routes, Yang et al. (2011) demonstrated that GO induce cytotoxicity via several pathways firstly generation of reactive oxygen species or direct physical toxicity via the interactions with the cell membrane lipid bilayer or indirect toxicity due to the adsorption of biological molecules. Furthermore, due to the hydrophobic surface, graphene can significantly interact with cell membrane lipids, causing toxicity (Zhang et al., 2010; Chen et al., 2012; Sanchez et al., 2012; Lammel et al., 2013; Nikodinovska et al., 2015). The present study investigates the genotoxicity of graphene oxide nanosheets in male albino mice via detecting the possible chromosomal aberrations occurring in bone marrow cells and evaluating the DNA damage (DNA fragmentation) occurring in lung tissue. In addition, determining oxidative stress parameters in lung tissue and evaluating the histopathological alterations.

2. Materials and methods

2.1. Preparation of graphene oxide nanosheets

Graphene oxide nanosheet was synthesized according to the modified Hummer method (Hummers and Offeman, 1958; Stahriany and Athawale, 2014). In briefly, 2 g of sodium nitrate powder (NaNO₃, Sigma-Aldrich, St Louis, MO) was dissolved in 100 ml concentrated sulfuric acid (H₂SO₄, 98%, Merck, Germany) under stirring. 2 g of graphite powder (99.9999%, Alfa Aesar, US) was added and stirred for 1 h to make a homogeneous mixture. Then, transfer it in ice bath (0 °C) with stirring. 12 g potassium permanganate (KMnO₄, 99.9%, Merck, Germany) was added gradually with stirring, and the mixture solution was kept at 0 °C in an ice bath. The mixture was stirred under cooling for 2 h. Then the ice bath was removed and the mixture was kept at 35 °C overnight with stirring. The temperature of the mixture was adjusted to 98 °C for 1 h while deionized water was added slowly (184 ml). Then, 560 ml deionized water was added to the suspension. 20 ml of H₂O₂, 30% (H₂O₂, 30%, Merck, Germany) was added. The reaction product was centrifuged and washed with deionized water repeatedly. Finally, the product was dried in a hot-air oven at 60 °C.

2.2. Characterisation of graphene oxide nanosheets

2.2.1. X-ray diffraction

Samples were air dried, powdered and used for XRD analysis. X-ray diffraction patterns were recorded in the scanning mode onan Xpert PRO PAN analytical instrument operated at 45 kV and a current of 30 mA with Cu Kα radiation (1.54 Å). The diffraction intensities were recorded from 35° to 79.93°, in 20 angles. The diffraction intensities were compared with the standard JCPDS files. The software gave the information about the structure.

2.2.2. Transmission electron microscope (TEM)

The morphology of GOs was examined using TEM (Technia, G20, 200 K, FEI, Netherland). GO were firstly sonicated for 5 min in deionized water and prepared by placing a droplet of the colloidal solution onto a carbon-coated copper grid and was allowed to dry for 15 min. Bright field imaging mode at electron accelerating voltage 200 kV using lanthanum hexaboride (LaB6) electron source gun was performed. Eagle CCD camera with (2k*2k) image resolution was used to acquire and collect transmitted electron images.

2.3. Experimental design

Ninety adult male albino mice (25 g) from the National Research Center (Giza, Egypt) were housed in plastic cages and kept on a 12 h light/dark cycle. The mice were fed commercial pelleted feed and given water ad libitum. The National Institutes of Health (NIH) guidelines for animal health and accommodation (Gordon, 1993; Smith et al., 2004; NIH, 2006, 2007) were supervised. A total of 90 male mature mice were randomly divided into six groups of fifteen mice per each, five groups treated with GO and one was kept as control. The five groups were injected by GO (i.p) at doses of 10, 50, 100, 250 and 500 μg/kg b.w once weekly in addition to the control group that was injected intraperitoneally with 0.2 ml saline solution. Five animals from each group were euthanized after 7, 28 and 56 days post treatment.

2.4. Chromosomal aberration assay

Bone marrow metaphases were prepared according to Yosida and Amans (1965) and Perston et al. (1987). Both treated and control animals were injected with 0.5 mg/kg colchicine 2 h before cervical dislocation. The colchicine is injected intraperitoneally to arrest the cell division at the metaphase stage. The femoral bone marrow was flushed with physiological saline solution (0.9% sodium chloride). The cells were centrifuged at 12000 rpm for 10 min and the supernatant was discarded. The cells were suspended in hypotonic solution of 0.56% potassium chloride, incubated at 37 °C for 20 min and then centrifuged for 10 min at 12,000 rpm and the supernatant was discarded and fixed at room temperature in methyl-acetic acid fixative (3:1 methyl alcohol: glacial acetic acid) for 10 min then centrifugation and washing were done twice in fixative. Finally the cells were resuspended in small amount of fixative; few drops of the cell suspension were dropped on a clean, cold slide stored in 70% ethyl alcohol and then dried on flame; after complete drying, slides were stained in 10% phosphate buffered Giemsa stain for 40–45 min, washed twice (5 min each) in the buffer, and then mounted with mounting media (per mount) and covered with clean and dry cover slips. Slides were examined microscopically at 1000 × magnification. At least 250 good metaphases of each animal were studied, scoring the different types of chromosomal aberrations structural and numerical aberration (John, 1973), with selection being based on uniform staining quality, lack of overlapping chromosomes and chromosome number (40 ± 1 chromosomes).

2.5. Single-sell gel electrophoresis (SCGE) analysis, comet assay

The DNA integrity and the incidence of DNA strand breaks or fragmentation (DNA damage) was detected using the alkaline comet assay according to the procedures described by Hartmann et al. (2003) and Liu et al. (2007) with minute modification. Briefly, a single cell suspension was prepared using mechanical dissociation and kept cells in phosphate-buffered saline to minimize cell aggregation. SCGE slides were prepared by mixing 10 μl of cell suspension with 90 μl 1% low melting, applying this to microscope slides precoated with 1% agarose, and covering with coverslip. After agarose gel has solidified, the slides were immersed in lysis solution consisting of (2.5 M NaCl, 100 mM EDTA (pH 8.0), 10 mM Tris, pH 10, supplemented with freshly added 1% Triton X-100, 1% N-lauroylsarcosine and 10% DMSO) then incubated overnight at 4 °C before SCGE. After completion of lysis step, the slides were placed for 20 min in an ice cold electrophoresis chamber containing alkaline electrophoresis buffer (1 mM EDTA + 300 mM NaOH, pH > 13) to produce single-stranded DNA and to express alkali-labile sites as single-strand breaks (unwinding).

Following alkali unwinding the electrophoresis was subsequently conducted for 20 min at 20 V/250 mA. Once the electrophoretic conditions have been established, the optimal electrophoresis duration depends on the extent of DNA migration desired in control cells, the range of responses being evaluated in cells from treated animals, the
Fig. 1. X-ray diffraction patterns of G (left) and GO.

2.6. Oxidative stress analysis

For measuring the SOD, CAT activities, GSH and MDA levels the type of cell being investigated and the electrophoretic equipment used. After electrophoresis, the samples were cut into small pieces and placed in 1 ml of 0.4 M HCl (pH 7.5) for 5 min then rinsed in distilled water for 5 min each. Then the slides were dehydrated with 1 ml absolute ethanol for 10 min in order to precipitate the DNA and just before visualization (Klaude et al., 1996).

Slides were stained with coating 50 μl of 20 μg/ml ethidium bromide. The slides were visualized with Leica epifluorescent microscope (Green filter: N2.1 with Excitation filter: BP 515–560, Dichromatic Mirror: 580, Suppression filter: LP 5920). The images of lung cells were digitized with a Leica DFC camera. DFC 280 supplied with the Leica DFC Twain software with Host application program Imaging™ to view and capture digital images. The most common magnifications used have been between 200× and 400×.

Using image analysis software TnTek CometScore™ freeware v1.5. As a minimum, 100 comets should be scored per tissue per animal, with 50 comets scored per replicate slide when five animals are used per group. Analyze individual comet images for several parameters including the percentage of DNA in the head and the tail (per cent migrated DNA), tail length and tail moment (fraction of migrated DNA multiplied by some measure of tail length). Of these, tail moment and/or tail length measurements are the most commonly reported, but there is much to recommend the use of percent DNA in the tail, as this gives a clear indication of the appearance of the comets and, in addition, is linearly related to the DNA break frequency over a wide range of levels of damage.

2.7. Histopathological examination

Lungs were removed carefully and fixed in 10% neutral buffered formalin, dehydrated in ascending grades of alcohol, cleared with xylene and embedded in paraffin, sectioned at 5 μm thickness and stained by H & E stain according to the method described by Bancroft et al. (1996), and followed by light microscopy examination.

2.8. Statistical analysis

Genotoxicity results were evaluated statistically according to statistical analysis; data were presented as mean ± SEM. Variables were statistically analyzed by one-way ANOVA test, using software SPSS (version 16). When differences were significant, LSD (Least Significant Difference) was performed to find the individual differences between groups. Statistical analyses were performed according to Snedecor and Cochran (1982).

3. Results

3.1. Characterization of graphene oxide nanosheets

3.1.1. X-ray diffraction

The crystalline structure and purity of graphene oxide and graphite were identified by X-ray diffraction (XRD). The degree of oxidation of graphite (G) and the interlayer spacing can be obtained. The XRD pattern of graphite as shown in Fig. 1(left) displays a characteristic peak at 2θ = 26.5415° indicating an interlayer spacing of 3.35566 nm with an index of (002) a sharp reflection plane. After oxidation with Hummer method, the characteristic graphite peak disappeared and replaced by a well-defined diffraction peak of GO as shown in Fig. 1(right) observed at about 2θ = 11.6341°, which corresponds to the (002) reflection of GO with inter-planar d-spacing 7.60023 nm which is much larger than that of pristine graphite (3.35566 nm).
3.1.2. High resolution transmission electron microscope (HRTEM)

Transmission electron microscopy (TEM) image showed that a wrinkled paper like structure of the large, ultrathin GO sheets and stacking of sheets as shown in Fig. 2.

3.2. Chromosomal aberrations observations

Statistical analysis of the total number and types of chromosomal aberrations (CAs) in different treated groups is shown in (Tables 1 - 3). The i.p administration of GOs to the male mice after 7, 28 and 56 days post GO treatment caused a significant increase in the total number of structural chromosomal aberrations in all different treated groups versus control group. A normal metaphase from bone marrow of control mice is shown in (Fig. 3A). All the concentrations of GOs induced mitotic division abnormalities. There were various chromosomal aberrations that had been observed in the bone marrow metaphase cells in all GOs treated groups that were dose and time dependent. Chromatid deletions were the most frequent structural chromosome aberrations observed (Fig. 3B) that showed a significant increase (p < 0.05) in all treated groups and different experimental periods compared with control group (Fig. 3A) and showed an increase in incidence with advancing dose. While, centric fusion was observed (Fig. 3C) but with a significant increase (p < 0.05) in dose 10 μg/kg at 7 days post injection, (100, 250 & 500 μg/kg after 28 days) and (250 & 500 μg/kg after 56 days). Centromeric attenuations were noticed (Fig. 3D) with no significant difference after 7 days after GOs administration versus control group while, doses groups of (50, 100, 250 & 500 μg/kg after 28 days) and (10 & 50 μg/kg after 56 days) showed a significant increase after GOs administration against the control group. Moreover, end to end association (Fig. 3E) showed non significant change after 7 & 28 days after GOs treatment, but only dose of 500 μg/kg group showed a significant increase after 56 days versus control. As well as fragments and chromatid breaks were detected during all experimental doses and intervals with no significant changes as compared to control group. In addition, ring chromosome was noticed (Fig. 3F) with significant increase at p < 0.05 in doses groups of (10, 50 & 100 μg/kg) after 7 days, but with non significant difference between the exposure time of 28 and 56 days in comparison with the control group. No significant changes in the numerical chromosomal aberrations were detected in all treated groups during different experimental intervals. The production of CA is a complex cellular process. According to the prevailing theories, structural (CA) result from (i) direct DNA breakage, (ii) replication on a damaged DNA template, (iii) inhibition of DNA synthesis, and other mechanisms such as topoisomerase II inhibition.

3.3. Comet assay observations in lung tissue

The single-cell gel electrophoresis (comet assay) allows detection of DNA fragmentation in single cells, and was initially used for DNA damage estimation. In this study, %DNA in comet head, percent DNA migration in the tail, comet tail length and tail moment were measured

<table>
<thead>
<tr>
<th>Types of chromosomal aberration after 7 days</th>
<th>Experimental groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (C)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Deletions</td>
<td>31</td>
</tr>
<tr>
<td>Centric fusions</td>
<td>29</td>
</tr>
<tr>
<td>Centromeric attenuation</td>
<td>5</td>
</tr>
<tr>
<td>End to end association</td>
<td>6</td>
</tr>
<tr>
<td>Fragments</td>
<td>0</td>
</tr>
<tr>
<td>Chromatid breaks</td>
<td>1</td>
</tr>
<tr>
<td>Ring chromosome</td>
<td>1</td>
</tr>
<tr>
<td>Total structural aberration (T.S.A.)</td>
<td>73</td>
</tr>
<tr>
<td>Polyplody</td>
<td>3</td>
</tr>
<tr>
<td>Endomitosis</td>
<td>1</td>
</tr>
<tr>
<td>Total numerical aberration (T.N.A.)</td>
<td>4</td>
</tr>
</tbody>
</table>

Data are Means ± SD.
* Significant at p < 0.05.
Table 2
Different chromosomal aberrations in bone marrow cells detected after 28 days of GOs different doses in male albino mice.

<table>
<thead>
<tr>
<th>Types of chromosomal aberration after 28 days</th>
<th>Experimental groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (C)</td>
</tr>
<tr>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

Deletions
- 21
- (4.2 ± 0.3)  
- 30
- (6.0 ± 0.3)  

Centric fusions
- 31
- (6.0 ± 0.3)  

Centromeric attenuation
- 9
- (0.6 ± 0.2)  

End to end association
- 5
- (1.0 ± 0.3)  

Fragments
- 2
- (0.4 ± 0.2)  

Chromatid breaks
- 2
- (0.4 ± 0.2)  

Ring chromosome
- 3
- (0.6 ± 0.2)  

Total structural aberration (T.S.A.)
- 66
- (9.4 ± 1.7)  

Polyplody
- 4
- (0.8 ± 0.3)  

Endomitosis
- 1
- (0.2 ± 0.1)  

Total numerical aberration (T.N.A.)
- 5
- (2.5 ± 0.4)  

Data are Means ± SD.
* Significant at p < 0.05.

Table 3
Different chromosomal aberrations in bone marrow cells detected after 56 days of GOs different doses in male albino mice.

<table>
<thead>
<tr>
<th>Types of chromosomal aberration after 56 days</th>
<th>Experimental groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (C)</td>
</tr>
<tr>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

Deletions
- 26
- (5.2 ± 0.4)  

Centric fusions
- 28
- (5.6 ± 0.5)  

Centromeric attenuation
- 3
- (0.6 ± 0.2)  

End to end association
- 2
- (0.4 ± 0.2)  

Fragments
- 1
- (0.2 ± 0.1)  

Chromatid breaks
- 1
- (0.2 ± 0.1)  

Ring chromosome
- 1
- (0.2 ± 0.1)  

Total structural aberration (T.S.A.)
- 62
- (8.8 ± 1.6)  

Polyplody
- 4
- (0.8 ± 0.3)  

Endomitosis
- 1
- (0.2 ± 0.1)  

Total numerical aberration (T.N.A.)
- 5
- (2.5 ± 0.4)  

Data are Means ± SD.
* Significant at p < 0.05.

as an indicators of DNA damage (Fig. 4A–C), showed formation of comets in lung cells treated with GOs, indicating genotoxic properties. Statistical analysis showed that GO was highly genotoxic towards lung tissue.

3.3.1. Percent of DNA damage in lung

The data shown in (Table 4) represent the % DNA damage. As expected, comparison between control group and GOs treated groups showed a significant increase (p < 0.05) in% DNA damage at all experimental periods. The extent of DNA damage along with number of comets was increased with increased doses of GOs. The elevation of% DNA damage in the lung was dose and time dependent.
3.3.2. %DNA in comet head, %DNA in tail, comet tail length and tail moment

The data expressed in Table 4 indicated a significant decrease (p < 0.05) in the lung %DNA in comet head after 7, 28 and 56 days of GOs administration groups as compared to control group. In addition, a significant increase (p < 0.05) was observed in %DNA in tail in lung nuclei of GOs-treated mice as compared to control animals, in a dose and time-dependent manner. In the same way, GOs induced a significant increase in comet tail length and tail moment in all treated groups during different experimental periods against control group as regarding to (Table 4) and (Fig. 4B–C).

3.4. Effect of GOs on oxidative stress indicators in mice lung tissue

The present study demonstrated that i.p administration of GOs to mice induced adverse effects on the antioxidant status of the lung. The

Table 4

<table>
<thead>
<tr>
<th>Experimental</th>
<th>Experimental Groups (Lung)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days- 7th days</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>%DNA in Head</td>
<td>83.3 ± 0.5</td>
</tr>
<tr>
<td>%DNA in Tail</td>
<td>16.7 ± 0.5</td>
</tr>
<tr>
<td>Tail Length (px)</td>
<td>3.5 ± 0.1</td>
</tr>
<tr>
<td>Tail Moment</td>
<td>1.4 ± 0.07</td>
</tr>
<tr>
<td>DNA Damage%</td>
<td>32</td>
</tr>
<tr>
<td>DNA Damage%</td>
<td>32</td>
</tr>
<tr>
<td>Experimental</td>
<td>Control</td>
</tr>
<tr>
<td>Days- 28th days</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>%DNA in Head</td>
</tr>
<tr>
<td>%DNA in Tail</td>
<td>17.6 ± 2.4</td>
</tr>
<tr>
<td>Tail Length (px)</td>
<td>4.9 ± 0.3</td>
</tr>
<tr>
<td>Tail Moment</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>DNA Damage%</td>
<td>36</td>
</tr>
<tr>
<td>DNA Damage%</td>
<td>36</td>
</tr>
<tr>
<td>Experimental</td>
<td>Control</td>
</tr>
<tr>
<td>Days- 56th days</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>%DNA in Head</td>
</tr>
<tr>
<td>%DNA in Tail</td>
<td>18.2 ± 2.1</td>
</tr>
<tr>
<td>Tail Length (px)</td>
<td>6.5 ± 0.9</td>
</tr>
<tr>
<td>Tail Moment</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>DNA Damage%</td>
<td>35</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM.

* Significant change at p < 0.05 versus control group.
Table 5
Effect of intraperitoneal treatment of graphene oxide nanosheets (10, 50, 100, 250 & 500 μg/kg body weight) on SOD (u/g wt tissue), CAT (u/gm tissue) activities, GSH (mg/gm tissue) and MDA (nmol/gm tissue) levels in lung of male albino mice after 7, 28 and 56 days of administration.

<table>
<thead>
<tr>
<th>Experimental Days</th>
<th>Experimental Groups (SOD)</th>
<th>Control</th>
<th>GO dose (μg/kg b.w.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>8.7 ± 0.3</td>
<td>4.7 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(−46.0%)</td>
<td>(−27.6%)</td>
</tr>
<tr>
<td>28</td>
<td></td>
<td>7.7 ± 0.3</td>
<td>8.2 ± 0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(6.5%)</td>
<td>(−31.2%)</td>
</tr>
<tr>
<td>56</td>
<td></td>
<td>7.1 ± 0.6</td>
<td>6.7 ± 0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(−5.6%)</td>
<td>(38.0%)</td>
</tr>
<tr>
<td></td>
<td>Experimental Groups (CAT)</td>
<td>7</td>
<td>17.3 ± 3.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(−50.9%)</td>
<td>(−21.4%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>28</td>
<td>12.6 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(−46.0%)</td>
<td>(−65.9%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>56</td>
<td>12.1 ± 1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(−28.9%)</td>
<td>(−40.5%)</td>
</tr>
<tr>
<td></td>
<td>Experimental Groups (GSH)</td>
<td>7</td>
<td>22.9 ± 1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(−43.0%)</td>
<td>(−20.2%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>28</td>
<td>11.4 ± 0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(−11.4%)</td>
<td>(16.7%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>56</td>
<td>12.2 ± 1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(9.8%)</td>
<td>(−16.4%)</td>
</tr>
<tr>
<td></td>
<td>Experimental Groups (MDA)</td>
<td>7</td>
<td>63.4 ± 4.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2.7%)</td>
<td>(51.7%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>28</td>
<td>84.1 ± 4.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(−6.1%)</td>
<td>(−10.0%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>56</td>
<td>70.9 ± 5.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(57.3%)</td>
<td>(64.5%)</td>
</tr>
</tbody>
</table>

The number of animals was 5 in each treated group.
Data are expressed as mean ± SEM.
* Difference with respect to control value.
* Significant change at p < 0.05 as compared to control group.

Data expressed from Table 5 indicated that large doses of GOs (250 & 500 μg) induced a marked and significant (p < 0.05) reduction in SOD activity after 28 and 56 days of GOs administration in all the examined tissues and groups as compared to control group, while a significant increase was detected at treated dose of 250 after 7 day post treatment. The results in Table 5 showed a continuous and significant reduction in CAT activity of lung after 28 & 56 days of all GOs treated dose groups as compared to control group. As deduced from Table 5 a significant reduction (p < 0.05) in GSH levels of the lung was noted after the administration of 10 & 500 μg/kg after 7 days of the treatment and for dose of 100 μg/kg after 56 days versus control group. Conversely, an elevation in MDA levels in the lung (Table 5) was recorded in all treated groups after 56 days at all time intervals as compared to control group. As well as, the same increase in MDA was noted after the administration of GO (50, 100, 250 & 500 μg/kg) after the 7 days versus control group.

3.5. Pulmonary histopathological alterations

In general the severity of pulmonary histopathological alterations was dose and duration dependent. Lesions from different treated groups were illustrated in Fig. 5. In 7th day exposure groups, there was a dose dependent increase in the intensity of the perivascular and peribroncholar mononuclear cell aggregations that became more diffuse to infiltrate the alveolar wall in addition to foam cell infiltration in alveolar lumen. Foam cell aggregations in the alveolar lumen were ranged from small individual foamy macrophages in dose groups of 10 & 100 μg/kg to dense cluster foam cells filling the alveolar lumen in higher dose treated groups (250 & 500 μg/kg). Moreover accumulation of eosinophilic edematous fluid in alveolar lumen was detected in higher dose exposure groups (250 & 500 μg/kg). These aforementioned pulmonary changes were detected after 28 and 56 days post exposure groups with increased in lesion severity with advancing dose and period of exposure. In addition to perivascular edema and fragmentation of the muscular layer of the small blood capillary wall was observed in 28 days post exposure groups and developed to sever hyalinization and fragmentation of the media of larger blood vessel associated with endothelial swelling and microthrombi formation. The microscopic examination of pulmonary tissue of control group revealed perivascular and peribronchial aggregation of small clusters of lymphocytes that was restricted to these areas and did not extend into the surrounding alveolar wall with no additional histopathological alterations were detected in this group.

4. Discussion

Graphene and graphene oxide nanosheets (GOs) represent the largest group of 2D nanomaterials used for biomedical applications including tissue engineering, drug delivery, bioimaging and biosensing (Chimene et al., 2015). Several studies were conducted on the toxicity of different concentrations of GOs in vitro and to a less extent in vivo studies. At the present work there was d-spacing increase of GOs in XRD analysis is due to the presence of abundant oxygen-containing functional groups on both sides of the graphene sheets caused by the oxidation. These results suggest that the layered GO has been exfoliated largely as previously confirmed by Ye et al. (2012). The genotoxic potential of GOs in the present study was represented by various types of structural chromosomal aberration that were dose and time dependent with significant increase, especially at higher doses at advancing time of GOs exposure this result agreed with (Patolla et al., 2010; Qiao
et al., 2013). The data were obtained from the present study confirmed that the interaction between GO and DNA was one of the main reasons for the mutagenic effect of GO. As well as, the secondary, indirect genotoxicity of the GOs is the result of interacting with cells, the generation of oxidative stress and reactive oxygen species (ROS) and that in turn will induce oxidative damage to the genetic material. Chang et al. (2011), Liu et al. (2013) confirmed the results of the present study as they recorded that GO and other carbon nanomaterials can induce the cellular generation of reactive oxygen species, which can also cause DNA damage. In addition, Graves and Velea (2000), Ren et al. (2010), Stueckle et al. (2016) indicated that the highly planar of GO may intercalate into the DNA helix and insert between base pairs of double strand DNA, similar to aromatic compounds. As well as, the interaction with several proteins involved in the DNA repair leading to inhibition of various DNA repair machineries as provided by Bhabra et al. (2009), Gupta et al. (2011), Cohignac et al. (2014). In the current work, the single cell gel electrophoresis (Comet assay) was used to detect the incidence of DNA fragmentation in the lung tissue. The present study indicated that i.p. administration of GOs to mice induced genotoxic effects on the DNA in lung in a dose and time dependent manner. Furthermore, our results demonstrated a marked and significant reduction in the percent of DNA in the head and a significant elevation in the percent of DNA in tail, the comet tail length and tail moment of lung after 7, 28 and 56 days of GOs administration. Previously, Lindberg et al. (2009), Berlo et al. (2012) attributed the genotoxicity of graphite nanofibres (GNFs) and carbon nanotubes (CNTs) to the fibrous nature of these carbon nanomaterials that induced a dose dependent increase in DNA damage (strand breakage) in human bronchial epithelial BEAS-2B cells by using comet assay. In the present study the pulmonary genotoxicity induced by GO may be attributed to both oxidative stress and mechanical injury, where GOs penetrate into cell nucleus destructing DNA. These findings were in agreement with Wang et al. (2013), Chng and Pumera (2015) who found that GO induce genotoxicity to human lung fibroblast cells by increased in tail length and tail DNA percentage which hinted at the extent of the DNA damage. Furthermore, De Marzi et al. (2014), Stueckle et al. (2016) showed that nano-GO was potent in induction of DNA damage in the lung (A549) cells using the comet assay. The present study indicated that i.p. administration of GOs to mice induced adverse effects on the antioxidant status of the lung in a dose and time dependent manner. The data of the present work indicated a marked and significant reduction in pulmonary SOD and CAT activities in mice after 28 and 56 days post treatment by higher doses of GOs when compared to control group. These results are in agreement with Ravichandran et al. (2011), Berlo et al. (2012) who observed a significant decrease in pulmonary SOD and CAT activity in Balb/c mice after inhalation exposure to SWCNTs. The reduction in SOD and CAT activities after administration of large doses of GOs may be attributed to the formation of ROS, which leads to the decrease of mitochondrial membrane potential in a dose dependent manner as indicated by Wang et al. (2011), Madani et al. (2013). In the present study there was an increased of SOD activity in the lung after administration of GO250 at 7th day post treatment that may be due to enzyme stimulation as a result of high levels of the superoxide anion radical as mentioned by Islam et al. (2014) and Patlolla et al. (2016) in GOs treated rats. Furthermore, Clichici and Filip (2015) proved that MWCNTs inhibit CAT activity due to the conformational changes and

Fig. 5. Lung of mice, (a–c) 7th day exposure time, (d–f) 28th day exposure time and (g–i) 56th day exposure time, (a) GO50 showing perivascular & peribronchiolar mononuclear cell aggregation (asterisk) with small focal aggregation of foamy macrophages (arrow) (×400). (b) GO100 treated group showing intense mononuclear cell aggregation in perivascular and peribronchiolar area (asterisk) that infiltrate the alveolar wall (×200). (c) GO250 showing accumulation of eosinophilic edematous fluid in the alveolar lumen (asterisk) (×400). (d) GO10 showing perivascular edema with fragmentation and disruption of the muscular layer of the small capillary wall (arrow) (×400). (e) GO50 showing perivascular mononuclear cell aggregation (asterisk). (f) GO250 showing intra alveolar foamy macrophage accumulation (arrow) (×400). (g) GO10 showing small focal foam cell aggregation in the alveolar lumen (circle) with perivascular and peribronchiolar mononuclear cell aggregation (×400). (h) GO100 showing fragmentation and hyalinized muscular layer (arrow) (×400). (i) GO500 showing intense intra alveolar aggregation of foamy macrophages with lymphocytic infiltration in the alveolar wall (×400) (b: bronchiole) (v: vascular capillary).
enzyme dysfunction. In addition, at the present work the reduction in GSH levels in lung after GOs administration with different doses and time intervals are in agreement with Khalilullin et al. (2015), Pacurarri et al. (2016) who noticed a decreased level of GSH after aspiration of MWCNTs or SWCNTs administration either by pharyngeal aspiration or inhalation in mice. Moreover, Liu et al. (2011) reported that intracellular GSH depletion following nanoparticles exposure is commonly interpreted as a marker for excess ROS production and also a result from the direct interaction of GSH at nanoparticle surfaces leading to the loss of GSH by adsorption, binding, or heterogeneous oxidation. Another finding which confirms the oxidative potential of GOs is MDA level in tissue. The data presented in this study indicated the elevation of MDA levels in lung of GOs treated groups that were dose and time dependent. These results agreed with Yang et al. (2008) who recorded an increase in MDA levels of lung of mice after exposure to SWCNTs for 90 days. The elevation of MDA levels may be attributed to the excessive ROS production after GOs administration causing the acceleration of lipid peroxidation and engendered membrane deterioration as mentioned by Lin et al. (2010) who observed an increase in MDA levels in tissues of mice after exposure to MWCNTs. Microscopic examination of the lung revealed various histopathological alterations that were dose and duration dependent, the lesions mainly of cellular inflammatory reaction and was detected in a lower dose of short duration exposure time and developed into lung edema in the higher dose at 7th days’ exposure time with foamy macrophage aggregation, these lesions were also detected by advancing exposure time. Perivascular and peribronchiolar aggregation of mononuclear cells extending into the alveolar wall with infiltration of neutrophils and macrophages in alveolar wall were observed in all GO doses treated groups, thus excluding the spontaneous occurrence of perivascular and peribronchiolar aggregation in control untreated mice as there was alveolar and vascular histopathological alterations accompanying this lesion. In addition, vascular changes were detected in higher doses that include perivascular edema, microthrombi and disruption of muscle layer of small blood vessels. The time and dose dependent manner of pulmonary lesions suggesting that GO can accumulate in lung tissue after i.p injection and induce lung pathology, similar results were observed by Liu et al. (2012) who found that GO can be cleared from blood after i.v injection of mice and accumulated primarily in lung for larger sized particles and liver for smaller sized ones, also Qu et al. (2013) observed that the increased accumulation of GO in lungs was likely due to GO-induced aggregation on blood cells within lungs, which were retained by capillaries in lungs. Previous researches confirmed the in vivo lung injury induced by GO that was injected via intravenous and direct tracheal instillation and clarified the possible mechanism of this injury, Kisseleva and Brenner (2008) & Manke et al. (2013) indicated that GO is highly toxic when administered directly to the lungs of mice causing severe and persistent lung injury and they attributed GO induced lung injury to the increase of mitochondrial respiration rate, generation of ROS thereby activating inflammatory and apoptotic pathways. Other studies proved the cytokines pathway of GO induced pulmonary inflammation and influx of neutrophils after direct instillation of GO into the lung of mice (Chen et al., 2012; Guo and Mei, 2014), while Li et al. (2013) reported the inflammatory response developed in lung after intratracheal instillation of GO and attributed lung edema in mice to the escaping of fluid from the capillaries into the interstitial and alveolar spaces and the loss of the lung activity to pump fluid out of the airspace. The formation of microthrombi in small blood capillaries induced by higher doses of GO after long exposure time confirming the thrombogenic nature of GO previously recorded by Wang et al. (2011) and Singh et al. (2011) who clarified that GO triggered severe lung thromboembolism and they considered GO as highly potent thrombogenic material after intravenous injection of GO into mouse.

5. Conclusion

It could be concluded from this study that GO induce chromosomal aberrations in bone marrow cells and DNA fragmentation in lung cells denoting the genotoxicity of graphene oxide nanosheets that were dose and time dependent. GO nanosheets have the potential to accumulate in lung tissue and induce pulmonary injury presumably by oxidative stress, elicited the inflammatory cascade in pulmonary tissue and induced lesions involving pulmonary capillary wall with increased capillary permeability and microthrombi formation denoting their pulmonary cytotoxicity that was dose and time dependent. Furthermore, in future, the dose of GO exposure must be taken in consideration and should be optimized and strictly controlled when used in biomedical applications.

References


