Experimental and Toxicologic Pathology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Experimental and Toxicologic Pathology

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

Graphene oxide nanosheets induced genotoxicity and pulmonary injury in mice

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ARTICLE INFO

Keywords:
Mice
Graphene nanosheets
Genotoxicity
Lung
Pathology

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 per each, five groups were intraperitoneally injected by GO 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. 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,

Abbreviations: A549, 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- 25-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; H_2O_2, hydrogen peroxide; H_2SO_4, sulfuric acid; HR-TEM, high resolution transmission electron microscope; i.p, intraperitoneal; i.v., intravenous; IBN, International Iberian Nanotechnology; JCPDS, Joint Committee on Powder Diffraction Standards; Kg, kilogram; $KMnO_4$, potassium permanganate; Kv, 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; $NaNO_3$, 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; SCGE, single cell gel electrophorsis; 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; TSA, 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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http://dx.doi.org/10.1016/j.etp.2017.03.002
Received 19 February 2017; Accepted 9 March 2017

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; Shahriary and Athawale, 2014). In briefly, 2 g of sodium nitrate powder ($NaNO_3$, Sigma-Aldrich, St Louis, MO) was dissolved in 100 ml concentrated sulfuric acid (H_2SO_4, 98%, Merck, Germany) under stirring. 2 g of graphite powder (99.9999%, Alfa Aeser, 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 ($KMnO_4$, 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 H_2O_2 30% (H_2O_2, 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. Characterization 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 X'pert PRO PAN analytical instrument operated at 45 kV and a current of 30 mA with Cu Kα radiation (1.54°A). The diffraction intensities were recorded from 35° to 79.93°, in 2θ 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 (Tecnia, G20, 200 Kv, 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 hexabromide (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 supervened. 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