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Apoptotic and anti-apoptotic genes transcripts patterns of graphene in mice

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ABSTRACT

Recent studies showed that a large amount of graphene oxide accumulated in kidney and liver when it injected intravenously. Evaluation of lethal and apoptosis gene expression in these tissues, which are under stress is very important. In this paper the in vivo dose-dependent effects of graphene oxide and reduced graphene oxide nanoplatelets on kidney and liver of mice were studied. Balb/C mice were treated by 20 mg/kg body weight of nanoplatelets. Molecular biology analysis showed that graphene nanoplatelets injected intravenously lead to overexpression of BAX gene in both kidney and liver tissues ($P \geq 0.01$). In addition these nanoparticles significantly increase BCL2 gene expression in both kidney and liver tissues ($P \geq 0.05$). Graphene significantly increase level of SGPT in groups 1 (220.64 ± 13), 2 (164.44 ± 9.3) in comparison to control group ($P \leq 0.05$). Also in comparison with control group (148.11 ± 10.4), ($P \leq 0.05$), the level of SGOT in groups 1(182.01 ± 12.6) and 2 (178.2 ± 2.2) significantly increased.

1. Introduction

Graphene, a two-dimensional nanomaterial, has surprisingly been applied in many fields, from industry to biology and medicine [1–2]. Because of unique properties of graphene, high hopes have been placed on its applications in photonics, electronics, optoelectronics and medicinal devices and sensors [3–6]. This wide spread application brings high attention towards this nanomaterial makes some essential question in people's minds, has it any side effect in environment? Evaluation of any possible toxicities of nanomaterial's which human have contact with them, is highly important and scientists should be aware of this matter. Cell toxicity and gene toxicity analysis were used by many researchers to analyze potential toxicity of graphene [7], in some studies in vitro concentration and size-dependent cytotoxicity of graphene in mammalian cells were investigated. The results showed that in some concentrations graphene cause toxicity in cells. Furthermore it is revealed that toxicity of graphene not only depends on concentration, but size and shape of nanomaterial are very important [7–10]. In our previous study we showed that graphene oxide and reduced graphene in a concentration-dependent manner cause some cyto and genotoxicity in mouse spermatozoa [11]. Very recently we studied that graphene nanosheets causes some abnormalities in mouse reproduction system when intravenously injected [12]. Furthermore, other works revealed toxicities of graphene in different cell lines [13]. Recently a research showed the biodistribution of graphene oxide in mouse body [14]. All these works mainly searched for any potential toxicity for graphene oxide, but there is a main question, in what molecular mechanism graphene makes toxicity in cells? Which genes are changed when toxicity occur? An important solution for this important gap is to evaluate gene expression of some genes, which are responsible for death and apoptosis in cells [15]. In this study, the potential toxicity of graphene oxide and reduced graphene in transcript level for two important genes in death and apoptosis pathway will be examined.

2. Material and methods

2.1. Experimental

2.1.1. Synthesis of GO

The modified Hummers' method was employed to graphite oxide synthesis from natural graphite powder (particle diameter ≤ 40 μm, Sigma Aldrich) [16,17]. Typically, 0.5 g graphite powder was added into H_2SO_4 at 80 °C for 5 h. Then, 0.5 g $NaNO_3$ was added and the mixture was stirred in an ice bath for 10 min. Then, 3.0 g $KMnO_4$ was slowly

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Table 1
Primer sequences for real-time reverse-transcription PCR analysis.

Gene	Sequence	Product Size (bp)
Bax	5′ GATCAGCTCGGGCACTTTAG-3′ 5′-TTGCTGATGGCAACTTCAAC-3′	101
BCL2	5′ AGGGTCTTCAGAGACAGCCA-3′ 5′ AGTACCTGAACCGGCATCTG-3′	124
GAPDH	5′-TCAATGAAGGGGTCGTTGAT-3 5′-CGTCCCGTAGACAAAATGGT-3′	125

added and the mixture was stirred for 2 h. After stirring, the mixture was cooled to room temperature in a water bath 35 °C. The obtained suspension was diluted by 40 mL deionized (DI) water and during the dilution, temperature of the suspension was fixed at <60 °C. Then, 3 mL H_2O_2 (30%) (diluted by 100 mL DI water) was added into the suspension. The residual acids and salts in the suspension were removed by filtering through a membrane filter (47 mm in diameter, 0.2 μm pore size, Whatman). The filtered graphite oxide material was dispersed in DI water to achieve an aqueous suspension with yellow-brownish color. The aqueous suspension was centrifuged (by an Eppendorf 5702 centrifuge with a rotor radius of 10 cm. Finally, GO suspension was obtained through sonication of the graphite oxide suspension.

To obtain a reduced GO (rGO), using ammonia solution, pH of GO suspension was adjusted ~9.0. Then, 100 μL of hydrazine solution (35%) was added to the suspension while stirring. After that, the suspension was refluxed at 90 °C for 3 h in an oil bath. The prepared rGO suspension was centrifuged & filtered and resuspended in DI water.

2.1.2. Material characterization

Graphene sheets surface topography was examined using atomic force microscopy (AFM, Digital Instruments NanoScope V) in tapping mode. To AFM analysis, GO samples were prepared by drop-casting a diluted suspension (~0.01 mg/mL) onto a cleaned Si(100) substrate. To investigate the chemical states of GO and rGO sheets X-ray photoelectron spectroscopy (XPS) was used. The data were acquired through a hemispherical analyzer equipped by an AlKα X-ray source ($h\nu$ = 1486.6 eV) operating at a vacuum better than 10–7 Pa. For more analyses, the XPS peaks were deconvoluted by using Gaussian components after a Shirley background subtraction. Also, quantitative elemental compositions were obtained using peak area ratio of the XPS core levels and the sensitivity factor of each element in XPS. Raman spectroscopy was performed at room temperature using a HR-800 Jobin-Yvon system supplied by a 532 nm Nd-YAG excitation source. The samples used in the XPS and Raman spectroscopy were prepared through casting conc. graphene suspension onto the Si substrate and removing the solvent at 20 °C in air for 30 min.

2.1.3. Determination of biochemical parameters

Blood and urine samples were collected and animals were sacrificed 1 h after last injection. The samples were centrifuged at 2500 ×*g* for 10 min and serums were collected and analyzed for uric acid and urea within 24 h using standard diagnostic kits (cell biolab kit). Urine for albumin determination was withdrawn from bladder immediately after sacrificing the animal. Albumin concentration was determined by the photometry.

2.1.4. Total RNA extraction and quantitative real-time PCR (qPCR)

Total RNA in kidney tissue was extracted using Rnasy kit according to the manufacturer's protocol (Qiagen). Extracted RNAs were quantified at 260 nm and stored at −70 °C until use. The RNAs were subjected to reverse transcription and then the cDNAs were quantified with real-time PCR using the Light Cycler DNA Master SYBR Green I protocol (Roche Diagnostics). The primers sequences are listed in Table 1.

2.1.5. Statistical analyses

All experiments were carried out in triplicate. The Student *t*-test and analysis of variance (ANOVA) were used for analysis of experimental data. SPSS 15 software was used for this analysis. Differences were considered significant at $P < 0.05$ or less.

3. Results

3.1. Results and discussion

A typical AFM image of the GO nanosheets is presented in Fig. S1A. The height profile shows thicknesses of ~0.8 nm for nanosheets (Fig. S1B). as reported in literature, the typical thickness of single-layer GO sheets is ~0.8 nm, which is ~0.44 nm thicker than the graphene (~0.36 nm). This is due to the presence of the oxygen functional groups on GO [16–19] and confirms the single-layer graphene synthesis.

XPS peak deconvolution of C(1 s) core levels of the GO and rGO sheets is presented in Fig. S1C. As shown in Fig. 1C, the peak fixed at 285.0 eV was attributed to the C—C and C═C bonds and the other deconvoluted peaks located at 286.6, 287.3, 288.3 and 289.3 eV were attributed to the oxygen-containing functional groups C—OH, C—O—C, C═O, and O═C—OH, respectively [16,17,20,21]. The XPS of rGO shows another deconvoluted peaks registered at 285.9 eV which can be attributed to formation of C—N bonds on surface of the hydrazine-rGO sheets [16,22,23]. The results show that reduction of GO into rGO, decreased the O/C ratio from 0.45 to 0.16.

To study the carbon structure of the GO and rGO sheets, Raman spectroscopy was utilized. The results of this study are presented in Fig. S1D. Comparison between D band of graphene and graphene oxide showed more intense D band of chemically reduced graphene instead of graphene oxide. It may be related to increasing the amounts of defects on the graphene sheets. Chemically deoxygenation of GO resulted in an increase in the intensity of the D band (~1355 cm^{-1}) and a decrease in the intensity of G band (~1585 cm^{-1}). The increase in the ID/IG intensity ratio from 0.92 for the GO to 1.19 can be assigned to decreasing the graphitic domain size and/or increasing the defects, due to formation of the C—N bonds [24–28].

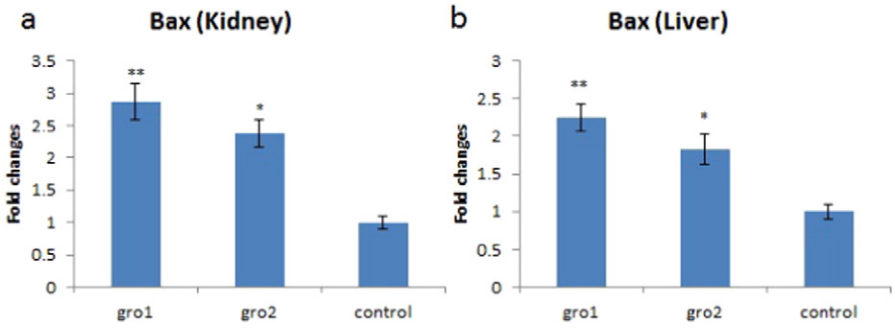

Fig. 1. Changes of Bax gene in kidney after treatments with GO and rGO. As figure showed both GO and rGO increase expression of this gene in mouse kidney.