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 20mg/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.

Keywords: Graphene, Apoptosis, Kidney, Liver, Mouse

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 nano sheets 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.

Material and methods

2. Experimental

2.1 Synthesis of GO

The modified Hummers' method was employed to graphite oxide synthesis from natural graphite powder (particle diameter $\leq 40\text{ }\mu\text{m}$, Sigma Aldrich) [16,17]. Typically, 0.5 g graphite powder was added into H_2SO_4 at $80\text{ }^\circ\text{C}$ 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 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 $<60\text{ }^\circ\text{C}$. 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, $0.2\text{ }\mu\text{m}$ 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.

2.2. 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 ($\sim 0.01\text{ mg/mL}$) 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 Al K α 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.

2.3. Determination of Biochemical Parameters:

Blood and urine samples were collected and animals were sacrificed 1h after last injection. The samples were centrifuged at $2500\times g$ 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.

2.4. 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.

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

2.5. 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.

Results:

3. Results and discussion

A typical AFM image of the GO nanosheets is presented in [Figure S1A](#). The height profile shows thicknesses of ~0.8 nm for nanosheets ([Figure 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(1s) core levels of the GO and rGO sheets is presented in [Figure S1C](#). As shown in figure 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 [16,23]. 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 Figure 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 ($\sim 1355\text{ cm}^{-1}$) and a decrease in the intensity of G band ($\sim 1585\text{ cm}^{-1}$). 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].

Quantification of BAX and BCL2 transcripts

Injection of graphene oxide in 20 mg/ml of mouse body weight intravenously increase BAX expression in kidney ($P \geq 0.01$), rGO also increase expression of this gene in kidney ($P \geq 0.05$), 2.86 and 2.37 fold changes respectively (Figure 1A). This increase in Bax transcripts not only appear in kidney, but also liver gene expression of Bax had a same pattern. Where Bax transcripts fold changes reached to 2.24 more than control group that it has significantly differences with control group ($P \geq 0.01$). The pattern for rGO groups is the same, rGO significantly increase the Bax transcripts ($P \geq 0.05$) that fold changes is 1.82 (Figure 1B).

BAX has been shown to be involved in p53-mediated apoptosis when cells are under stress [29]. Previous studies revealed graphene nanoparticles with high sharp edges induce oxidative stress

Figure 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.

in cells [30] that is one of the important mechanisms of toxicities of this nanomaterial. Our results confirm these, as graphene induce stress in cells, this stress leads to activation of some apoptotic genes like BAX, which showed that graphene increase transcripts of this gene, therefore BAX gene can be suggested as a marker gene for evaluation of particles toxicity in molecular levels.

Quantitative real-time PCR showed a significant increase in transcript level of kidneys BCL2 gene in groups 1 (1.65 ± 0.18), 2 (1.41 ± 0.18), in comparison with control group ($P < 0.05$) Figure 2a. A same expression pattern for this gene showed in mice livers when injected by GO and rGo, in GO-treated group BCL2 gene up regulated and its transcripts reached to 1.54 ± 0.14 , and rGo-treated group increase transcripts up to 1.33 ± 0.13 Figure 2b. Bcl-2 which is member of BCL2 family genes that has been identified as an important anti-apoptotic protein and is classified as an oncogene. When a factor induce cells towards death BAX gene up regulated, and plays important role in this pathway, BCL2 also up regulate as well, but this protein try to inhibit apoptosis in cells, so when cells are under stress these two important gene express and identify that whether cells come towards death or not [31]. The ration BAX/BCL2 is the most important things in

expression of these genes, when BAX express more than BCL2, cells will die, but if expression of BCL2 be more than BAX cells will be oncogene cells, this ratio in normal cells is always 1 [31]. After analyzing the data, it was clear that GO and rGO induce apoptosis in kidney and liver cells, BAX/BCL2 ratio in kidney cells was 1.73 for GO-treated group, and 1.64 for rGO (Figure 3). When GO injected in body, the large amount these particles accumulated in kidney cells, which leads to increase of reactive oxygen species (ROS) in this organ [12]. Sour data approved this result and introduce the main molecular mechanism of toxicity in cells.

Figure 2. Changes of Bax gene in kidney (a) and liver (b) after treatments with GO and rGO. As figure showed both GO and rGO increase expression of this gene in mouse kidney.

Figure 3. Bax/BCL2 ratio in a) liver and b) kidneys mice when treated by GO and rGO.

Cytotoxicity studies of graphene mainly DNA damage and gene expression, may be related to physico-chemical interactions of graphene with the cells. Experimental results indicate that morphology, surface functional groups and surface charge affects on the graphene toxicity as well as DNA damage and gene expression. Also, graphene can directly interact with different genes encoding important proteins and enzymes or indirectly ROS cause DNA damage resulted in inhibition or activation of specific enzymes. However to obtain a full view on the graphene action inside the cell more studies are required.

Biochemical Parameters:

GO and rGO administration leads a significantly increase in uric acid and urea levels. The baseline levels of uric acid and urea in mouse serum were measured as 4.1 ± 0.31 and $29.0.30$ mg/dl, respectively. A significant increase in serum levels of uric acid and urea (6.61 ± 0.51 and 43.9 ± 2.71 mg/dl) ($P < 0.05$) was detected after GO injection. In addition, the injection of 20mg/kg of rGO increase the uric acid and urea levels ($P < 0.05$) (Figure 4). damaged to kidney cells which is in agreements with other works which graphene accumulated in kidney and damage to cells.

Figure 4. The level of uric acid (a) and urea (b) in GO (group 1) and rGO (group2) treated mice. Go and rGO significantly increase the level of uric acid and urea in mice when compare with control group (saline injected) ($P < 0.05$).

These results showed that when graphene injected, the amount of particles accumulated in mice kidney and We then carried out the serum biochemistry assay for mice i.v. injected with GO and rGO. The liver function parameters including alanine aminotransferase (SGOT) and alkaline phosphatase (SGPT) were tested in the serum biochemistry assay. 72h after injection of GO and rGO the level of SGOT evaluated to 182.01 ± 12.6 and 178.2 ± 2.2 respectively. These differences were significant when compare with control group (148.11 ± 10.4) (Figure 5). The level of SGPT also reached to 220.64 ± 13 in GO-treated group and 164.44 ± 9.3 in rGO-treated group. Any changes in these important biochemical factors of liver are associated with hepatic injury. This increase in biochemical factors, which usually exhibit hepatic toxicity confirm our molecular analysis that showed the BAX gene was up regulated and signifies apoptosis in hepatic cells.

Figure 5. The level of a) SGPT and b) SGOT in serum of mice treated with GO (group 1) and rGO (group 2). GO significantly increase SGPT level ($P < 0.01$).

4. Conclusion

In this study, we have systematically investigated the in vivo toxicity of GO and rGO when intravenously injected. Our results showed that GO after injection of graphene, the kidney and liver cells were damaged and their function disturbed where a significant toxicity was noticed in serum biochemistry. Also after gene expression analyzing, apoptotic molecular markers like BAX was up regulated. Our results suggest that nanomaterials such as graphene via changes in gene profile expression causes toxicity.

Acknowledgment

The authors would like to thank the Iran National Science Foundation (INSF) for financial support of the work for the facilitation of the process to conduct this study. The authors would like to thank the National Institute of Genetic Engineering and Biotechnology and Sharif University of Technology and Islamic Azad University for the facilitation of the process to conduct this study.

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