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Synthesis and cyto-genotoxicity evaluation of graphene on mice spermatogonial stem cells

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ABSTRACT

The present study analyzed the dose-dependent cyto- and genotoxicity of graphene oxide and reduced graphene oxide on spermatogonial stem cells (SSCs) for the first time. The results showed that graphene oxide significantly increased oxidative stress at concentrations of 100 and 400 μg/ml, while low concentrations did not have a significant effect. In addition, according to the MTT assay, the cell number decreased in high-concentration (100 and 400 μg/ml) graphene oxide-treated samples compared to untreated cells. However, a reduced graphene-treated sample demonstrated a significant increase in cell number. Moreover, microscopic analysis found high concentrations of graphene nanosheets in cell culture medium that reduced the number of colonies and colony forming cells. We conclude that a high concentration of graphene can be toxic to SSCs. However, such toxicity can be reduced by the surface modification of graphene nanomaterials.

1. Introduction

Nanomaterials have been recently applied in fields such as medicine, agriculture, and industry, as well as the environment. Regarding medicine, different types of nanoparticles have been used in cancer therapy, wound healing, infectious diseases, and drug delivery [19,26,34,35]. Graphene is a new 2-dimensional nanomaterial with special properties, including a large surface area, high electrical and thermal conductivity, and increased mechanical properties and biocompatibility. Additionally, large scale production of graphene is low in cost. These properties have caused graphene to be an applicable material in electronics and medicine [28,42]. Consequently, graphene nanoparticles are currently employed in drug delivery, photo-thermal cancer therapy, biosensing, biocompatible scaffolds, bio-imaging, and anti-microbial components [50,53,51,54,18,11,52,10,55,23,40]. Increasing interest in the use of graphene nanoparticles might increase the risk of human exposure to such materials in the environment. Although research on the technical and biomedical applications of graphene, graphene oxide and graphene derivative nanomaterials is expanding rapidly, there has been insufficient research regarding the possible effects of biohazardous graphene nanomaterial on biological systems and their intrinsic toxicity [38]. Accordingly, the identification of graphene toxicity could result in the generation of safer nanoparticles by manipulating factors, such as size and dose. A number of studies have examined the toxicity of graphene nanoparticles on cells. The mechanisms that underlie the cytotoxic effects lead to the impairment of mitochondrial activity [39,5], plasma membrane damage [39,5,50,53], induction of oxidative stress [50,53,49,29,13], DNA damage [2] and apoptotic and/or necrotic cell death [39,50,53,29,47].

Most of the cytotoxic studies have been performed on somatic cells, not spermatogonial stem cells (SSCs) [7,8,50,53,46,22]. SSCs are unique cell populations that can self-renew and differentiate into germ cells. The biomedical applications of graphene may not be realized unless the potential toxicity of this nanomaterial to humans and other biological systems is thoroughly ascertained. Therefore, the present study explores the cyto- and genotoxicity of graphene nanoparticles on SSCs. The MTT assay was utilized to study the viability of SSCs treated with graphene nanoparticles. Dose-dependent cyto-genotoxicity of graphene nanoparticles was

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reported for the first time. The comet assay was also employed to study DNA damage and chromosomal aberrations. In addition, the oxidant-sensitive dye assay was used to monitor the level of reactive oxygen species (ROS).

2. Material and methods

2.1. Synthesis of Graphene Oxide

The modified Hummers' method was applied to synthesize graphite oxide (GO) from natural graphite powder (particle diameter ≤40 μm, Sigma-Aldrich) [24,27]. Typically, 0.5 g of graphite powder was added into H_2SO_4 at 80 °C for 5 h. Then, 0.5 g of $NaNO_3$ was added and the mixture was stirred in an ice bath for 10 min. Next, 3.0 g of $KMnO_4$ was slowly added and mixed for an additional 2 h. Once the $KMnO_4$ was dissolved, the mixture was cooled to room temperature in a 35 °C water bath. The suspension was diluted by adding 40 ml of deionized (DI) water. During the dilution, the temperature was fixed at <60 °C. Then, 3 ml of H_2O_2 (30%, diluted in 100 ml DI water) was added to the suspension. The residual acids and salts in the suspension were removed by 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 a yellowish-brown color. After centrifugation of the aqueous suspension (Eppendorf 5702 centrifuge, 10 cm rotor radius), the GO suspension was obtained after sonication of the pellet.

To obtain reduced GO (rGO), the pH of the GO suspension was adjusted to ~9.0 using an ammonia solution. Then, 100 μl of a hydrazine solution (35%) was added to the suspension while stirring. Next, 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

The surface topography of the graphene sheets was examined using atomic force microscopy (AFM, Digital Instruments NanoScope V) in tapping mode. For 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 the GO and rGO sheets, X-ray photoelectron spectroscopy (XPS) was used. The data were acquired through a hemispherical analyzer equipped with an Al Kα X-ray source ($h\nu = 1486.6$ eV) operating at a vacuum greater than 10–7 Pa. For further analyses, the XPS peaks were deconvoluted using Gaussian components after a Shirley background subtraction. The quantitative elemental compositions were obtained using the peak area ratio of the XPS core levels. 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 for XPS and Raman spectroscopy were prepared by casting a graphene suspension onto a Si substrate and removing the solvent at 20 °C in air for 30 min.

2.3. Isolation of spermatogonial stem cells

One-week-old BALB/c inbred mice were anesthetized and sacrificed. Testicles were removed and placed in phosphate buffer saline (PBS) containing antibiotics. Excess tissue was removed, and the samples were washed with PBS. The testicles were chopped with a surgical blade in an enzymatic digestion mixture containing hyaluronidase, DNaseI and collagenase [21]. Next, the separated cells were co-cultured with mouse fibroblast cells in DMEM containing 20% fetal bovine serum (FBS) with 50 IU/ml penicillin and 50 μg/ml streptomycin. Cells were observed for colony formation.

2.4. Treatment of SSCs with graphene nanoparticles

Treatment of the SSCs was performed by adding GO/rGO nanoparticles to the cultured cell medium to a final concentration of 1, 10, 100 and 400 μg/ml. An untreated culture was used as the control. Microscopic changes of the SSCs after treatment with nanoparticles were visualized using an inverted microscope (Nikon).

2.5. MTT assay

The viability of graphene nanoparticle-treated SSCs was assayed by the MTT test. The cells were briefly cultured in a 96-well plate and incubated for 3 h with MTT solution. DMSO was added to the wells to solubilize formazan particles, and the absorbance was read at 580 nm using an ELISA reader (Microplate reader labsystem multiscan).

2.6. ROS assay in spermatogonia

In the treated cells, the amount of ROS was measured using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Sigma Co.). After treatment, the cells were incubated at 37 °C and 5% CO_2 for 30–60 min with 10 mM DCFH-DA. The cells were enzymatically detached by trypsin and washed three times with PBS. The resulting cell suspension was centrifuged for 5 min at 1000 RPM. The conversion rate of DCFH to dichlorofluorescein (DCF) was measured using a fluorescence spectrophotometer with a 480-nm excitation and 520-nm emission wavelengths.

2.7. Membrane integrity

Membrane integrity was tested by acridine orange staining to observe apoptotic morphological changes. The GO-treated SSCs were incubated with 100 μg/ml acridine orange dye for 30 min in a CO_2 incubator. After washing with PBS three times, the SSCs were monitored at 200× magnification using a fluorescent microscope (Nikon).

2.8. Comet assay

DNA damage in the treated SSCs was investigated using a comet assay as previously described by Tice et al. [45]. Frosted slides were dipped in molten agarose and dried at 50 °C. Cells were embedded in slides coated with double layers of agarose. The slides were then immersed in a lysis solution containing NaCl (2.5 M), EDTA (100 mM), *N*-lauroylsarcosine (1%) and trizma (10 mM) at a pH of 10 for 1 h. Triton X-100 (1%) was added to the cells. The slides were incubated in electrophoresis buffer (pH 13) for 20 min to produce ssDNA. The solution was incubated in EDTA (1 mM) and NaOH (300 mM). To identify the possible comet, gel electrophoresis was performed on the ssDNA in the slides at 1.0 V/cm. After neutralization with trizma buffer, ethidium bromide was added for comet visualization. Finally, comet evaluation was based on the percentage of DNA in the tail.

2.9. Statistical analysis

The obtained data were studied via analysis of variance using the SPPP statistical software (*ver*. 19). Additionally, comparisons between means were performed using Duncan. The level of significance was $P \leq 0.05$. All experiments were replicated three times.