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Synthesis of Graphene Oxide and *in vitro* Evaluation of Its Cytotoxic Effect

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ABSTRACT

Objective: Graphen oxide, a carbon-based material, is considered as a potential material for many biomedical applications such as cell labeling, cell imaging, biosensors, drug release studies, due to its physical and chemical properties. Graphene oxide can be synthesized by more than one method. In this study, it was aimed to evaluate the cytotoxic effects of the graphenoxide samples we synthesized.

Methods: Synthesis of graphene oxide was done by Hummers method. The cytotoxic effects of graphene oxide were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay in human embryonic kidney cells (HEK293). After 24 hours, 48 hours and 72 hours of graphene oxide incubation at different doses, the IC50 values were calculated which 50% percentage of cell viability.

Results: After 24 hours, 48 hours and 72 hours, IC50 values were measured as 206.18 µg/mL, 108.98 µg/mL, and 55.54 µg/mL, respectively. It was determined that the IC50 value decreased as the incubation time increased.

Conclusion: It was determined that the cytotoxic effect of the synthesized graphene oxide varies depending on the exposure time.

Keywords: Graphene, graphen oxide, HEK293, toxicity

INTRODUCTION

Carbon is one of the most abundant elements in the earth's crust. Carbon-based materials have many different application areas due to their high chemical resistance ability, efficient mechanical properties, low weight characteristics and high dispersibility in the body. Materials with different properties are formed by the bonding of carbon atoms to each other in different ways. Multilayer graphene, graphene oxide (GO), carbon nanotubes, nano diamonds and reduced GOs are carbon derivatives used in biomedical systems (1).

Graphene was first described as crystal graphitic films by Nobel laureates Andre Geim and Konstantin Novoselov. Graphene, which has a two-dimensional structure, is 1 atom thick and has the appearance of a honeycomb formed by the bonding of sp^2 hybrid carbon atoms. Graphenes, specific surface area (2630 m^2G^{-1}), high charge mobility (200,000 $cm^2 V^{-1}\cdot S^{-1}$), high Young's modulus (~1.0 TPa), high thermal conductivity (~5000 $Wm^{-1}\cdot K^{-1}$) and high optical transmittance (~97.7%) have unique thermal, electrical and mechanical properties (2-4). Because of these properties, the use of graphene-based materials in various biomedical applications, including tissue engineering, biosensing, gene delivery and cancer therapy, is increasing day by day (1,5).

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GO is a chemically modified form of graphene and graphene is hydrophobic while GO is hydrophilic. This feature of GO has made it suitable for use as a drug delivery agent, cellular imaging probes and biosensors in biological systems (1,6,7). GO contains both aromatic (sp^2) and aliphatic (sp^3) domains. Its surface is even wider to allow electrostatic interaction, and it allows drug binding. Therefore, it is also compatible with drug release studies (5,8). The functionality of GO as a toxic or biocompatible material depends on its composition, size, surface, shape, functional groups, charge, coating and solute medium. In the literature, more than one method is used for the synthesis of GO (9,10).

In this study, it was aimed to synthesize GO and evaluate the cytotoxic effect of synthesized GO in embryonic kidney cells.

METHODS

Graphen Oxide Synthesis

GO synthesis was carried out by the modified Hummers method (11). All chemicals used in the synthesis of GO were used in analytical purity: Sulfuric acid (H_2SO_4) (MERCK, 7664-93-9, Germany), phosphoric acid (H_3PO_4) (MERCK, 7664-38-2, Germany), Graphite, Potassium Permanganate ($KMnO_4$) (ISOLAB, 7722-64-7, Germany), hydrogen peroxide (H_2O_2) (30% wt) (MERCK, 1085972500), hydrochloric acid (HCl) (38% wt) (ISOLAB, 7647-01-0, Germany), anhydrous ethanol (ISOLAB, 64-17-5, Germany), sodium hydroxide (NaOH) (MERCK, 106498.1000, Germany).

For the synthesis of GO, 360 mL of H_2SO_4 and 40 mL of H_3PO_4 were placed in a beaker and mixed at constant temperature at 200 rpm. Then 3 g of graphite and 18 g of $KMnO_4$ were slowly added to the solution. This reaction was stirred at 40-45 °C for 16 hours. The suspension, which was stirred for 16 hours for the synthesis of GO, was transferred to a beaker containing 400 g of ice and mixing was continued. While the suspension was mixed with ice, 3 mL of H_2O_2 (30% by weight) was added dropwise. The resulting mixture was centrifuged at 3000 rpm for 45 minutes. After centrifugation, the supernatant (acid) was discharged into the waste bin. GO was obtained by washing the pellets and then drying them in an oven.

Characterization of Graphen Oxide

In order to determine the functional groups in the obtained GO structure, Fourier Transform Infrared (FT-IR) analysis was performed in the range of 4000-400 cm^{-1} using FT-IR spectroscopy (Nicholet FT-IR Spectrometer). The images of the samples were taken with the Transmission Electron Microscope (Jeol JEM 2100 plus).

Cell Culture

Commercially available human embryonic kidney cells HEK293T (CRL-1573) (The American Type Culture Collection-ATCC; Manassas, VA, USA) were cultured in a 37 °C, 5% CO_2 incubator in the medium prepared with 89% Dulbecco's modified eagle medium, 9% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic solution (100 U/mL penicillin and 100 U/mL streptomycin). When the cells reached 70-80% density, they were first washed with Dulbecco's phosphate buffer saline and then removed from the flask with 0.25%. Trypsin-EDTA. The trypsin-EDTA-cell suspension was centrifuged at 120xg for 5 min. After centrifugation, the supernatant was discarded and fresh medium was added to the cell pellet. Cells were incubated by seeding in 96-well plates ($1x10^4$ cells/well). All chemicals were commercially available (Euroclone S.p.A.; Via Figino-Italy).

Application of Graphene Oxide and MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) Test

The MTT test was used to measure cellular metabolic activity in the evaluation of cell viability, proliferation and cytotoxicity. This colorimetric assay uses metabolically active cells to convert the yellow tetrazolium salt MTT into purple formazan crystals. NAD(P)H-dependent oxidoreductase enzymes in living cells convert MTT to formazan. Using dimethyl sulfoxide (DMSO) to dissolve insoluble formazan crystals, the absorbance of the resulting colored solution is measured in a spectrophotometer. Darker wells contain more metabolically active living cells, and the color is lighter in wells with reduced cell viability (12,13).

In our study, in order to determine the effective dose (IC_{50}) of GO, cells were seeded in 96-well plates at $1x10^4$ cells/well. Twenty four hours after cell cultivation, control group was treated with fresh medium, experimental groups were treated with GO at different concentrations (1000-500-250-125-62.5-31.25-15.62-7.81-3.90 µg/mL). It was incubated for 24, 48 and 72 hours. At the end of the experiment, the medium was removed from all wells. 100 µL of fresh medium and 10 µL (5 mg/mL) of MTT solution were added to the wells and the cells were incubated for 3 hours. At the end of the incubation period, 100 µL of DMSO was added to each well and absorbance values were measured at 570 nm in an ELISA microplate reader (Multiskan GO-Thermo). By using optical density values, dose (IC_{50}) values for GO, which reduced cell viability by 50%, were determined with the GraphPad Prism 9 program.

Measurements were made in 3 repetitions and the results were expressed as mean values, statistical evaluation was not made.

RESULTS

Fourier Transform Infrared Spectroscopy Analysis

FTIR Spectra are given in Figure 1. Between 3000-3500 cm^{-1}, a broad peak is observed, corresponding to the stretching and bending vibration of the OH groups of water molecules adsorbed on GO. This peak indicates that GO has a strong hydrophilic structure. The characteristic peaks at 1735 cm^{-1} indicates the carboxyl C=O group, and the peak at 1630 cm^{-1} indicates the aromatic C=C group. The absorption peaks at 2920 cm^{-1} and 2850 cm^{-1} correspond to the stretching vibrations of CH_2. The absorption peaks at 1200 cm^{-1} indicate the stretching vibration of the C-O bond of the carboxylic acid. It shows the presence of absorption bonds in the range of 2925 cm^{-1}, 2119 cm^{-1}, 1768 cm^{-1}, 1631 cm^{-1}, 1380 cm^{-1}, 916 cm^{-1} and 534 cm^{-1}.

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Morphological Characterization

Looking at Figure 2, it is seen that GO has a partially uniform transparent morphology due to its characteristic monolayer feature. Due to the large specific surface area formed by the oxygenated functional groups during the oxidation process, numerous bends and irregular wrinkles are observed on the surface (14).

MTT Results

HEK293 cells were treated with different concentrations of GO (1000-500-250-125-62.5-31.25-15.62-7.81-3.90 µg/mL). It was observed that GO decreased cell viability depending on time and dose (Figure 3). IC_{50} values were calculated as µg/mL and given in Table 1.

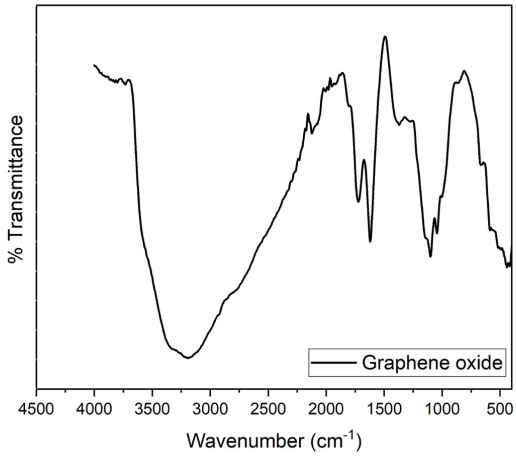

Figure 1. FT-IR spectroscopy of graphene oxide
FT-IR: Fourier Transform Infrared

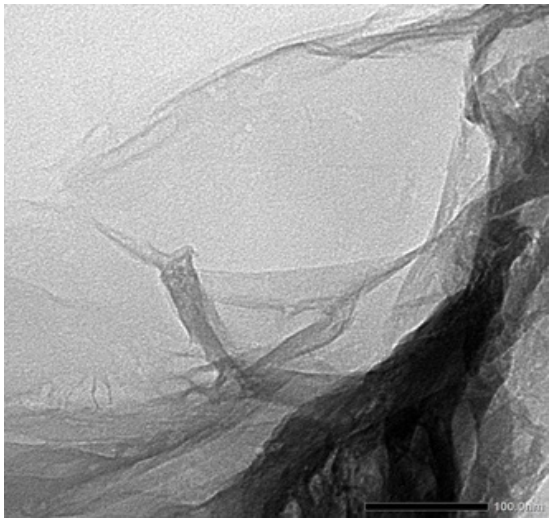

Figure 2. TEM photograph of graphene oxide
TEM: transmission electron microscope

DISCUSSION

Since carbon is one of the most common elements in nature, it is known that the biocompatibility of carbon materials is higher than other inorganic materials. In particular, graphite has been used in our daily lives for hundreds of years without critical toxicity issues. Due to the unique physical and chemical properties of graphene, which is a single layer of graphite, its use in biological studies is increasing day by day (1,2,3,7).

In this study, it was aimed to determine the cytotoxic effect of the synthesized GO on the viability of human embryonic kidney cells. For this purpose, the effects of 1000-500-250-125-62.5-31.25-15.62-7.81-3.90 µg/mL concentrations of GO on HEK293 cells were evaluated by MTT analysis, which was evaluated depending on mitochondrial activity. According to the results obtained, cell viability was found to be over 75% at the incubation period of 24 and 48 hours and at the concentrations of 125-62.5-31.25-15.62 µg/mL. When GO incubation was 72 hours, cell viability decreased below 65% at values above 62.5 µg/mL concentration. IC_{50} values showing 50% viability concentrations for 24, 48 and 72 hour incubations were calculated as 206.18 µg/mL, 108.98 µg/mL and 55.54 µg/mL, respectively. It was determined that when the incubation period with GO increased, mitochondrial damage increased, thus negatively affecting cell viability.

Today, there are many *in vitro* and *in vivo* studies investigating the biological effects of different graphene derivatives (4,6,9). Zhang et al. (15) found in their study on PC12 cells that the cytotoxic effect of graphene derivatives with different structures was directly related to the graphene structure. In another study investigating the effects of graphene, Wu et al. (16) showed that nano zinc oxide did not show toxic effects on cell viability, oxidative stress, mitochondrial depolarization and membrane damage in A549 cells when bonded with graphene.

Table 1. IC_{50} values for 24, 48 and 72 hours incubations in HEK293 cells treated with graphene oxide

	24 hours	48 hours	72 hours
IC_{50} value	206.18 µg/mL	108.98 µg/mL	55.54 µg/mL

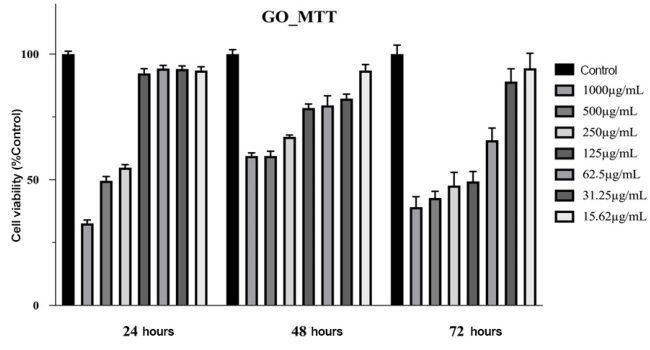

Figure 3. Time and dose dependent effect of graphene oxide on HEK293 cells

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GO, one of the graphene derivatives, is interesting for its use in biological systems due to its hydrophilic structure. Due to the wide application area of GO, a comprehensive understanding of the cytotoxicity of GO is required for the safe and sustainable development of GO-based technologies (6). In this study, we aimed to observe the effect of GO, which we synthesized by using the modified Hummers method, on HEK293 cells.

Wang et al. (17) evaluated the biocompatibility of GO, which they synthesized by using the modified Hummers method, in human fibroblast cells and mice. While the application of GO at a dose of less than 20 µg/mL did not show toxicity in human fibroblast cells, it showed significant cytotoxic effects such as decreased cell adhesion, inducing apoptosis, penetrating into lysosomes, mitochondria and cell nuclei at doses above 50 µg/mL. On the mice, it was determined that low and medium doses of GO did not show significant toxicity, but at high doses (0.4 mg) it exhibited chronic toxicity with accumulation in the liver, spleen and kidney, and caused lung granuloma. In a study on human erythrocytes, it was reported that GO showed dose-dependent hemolytic activity, and the particle size of GO samples also played a critical role in hemolic activity (18). In a study investigating the effects of nanoGO on cardiomyoblast cell line, incubation with 20, 40, 60, 80 and 100 µg/mL nano-GO for 24 hours caused mitochondrial hyperpolarization, free radical production, and DNA damage at concentrations of 40, 60, 80, 100 µg/mL (19). Lammel et al. (20) showed that the cytotoxic effect of GO was dose-dependent through plasma membrane damage, and that GO damaged the structural integrity of the plasma membrane by establishing a strong physical interaction with the phospholipid bilayer. They also reported that GO could penetrate the plasma membrane, resulting in altered cell morphology and an increased number of apoptotic cells (20).

Ünal et al. (4) investigated the effect of different graphene derivatives in their study and showed that the viability of A549 human lung epithelial carcinoma cells was 17% even at the highest dose of 1000 µg/mL of GO. GO showed the greatest effect on MCF-7 breast cancer cells at the lowest dose tested, 1.96 µg/mL, and it was found to cause 11% death. However, they reported that the same graphene derivatives did not show a significant cytotoxic effect in healthy 293-T human embryonic kidney epithelial cells (4). However, Hu et al. (21) stated that the effect of GO was greatly attenuated by incubation with 10% fetal bovine serum, due to the extremely high protein adsorption ability of GO. In another *in vitro* study, it was reported that low-dose GO had no effect on the morphology, viability and membrane integrity of the A549 cell line, but it could cause a dose-dependent oxidative stress and cause a decrease in cell viability at high doses (22). Similar to the studies, in our study, it was observed that cell viability of HEK293 cells that apllied different concentrations of GO decreased depending on time and dose.

Study Limitations

Molecular studies are needed to determine the cytotoxic effect of GO and to elucidate its interaction with the cell. In this sense, our study can be considered as a preliminary study to investigate the effect of GO.

CONCLUSION

As a result, more studies are needed to increase the usability of graphene derivatives synthesized by different methods in biomedical applications and to evaluate long-term toxicity/ biocompatibility data.

Ethics Committee Approval: This study does not require ethics committee approval.

Informed Consent: This study does not require patient consent.

Peer-review: Internally peer-reviewed.

Author Contributions: Concept - N.P.Ö., D.D.E.; Design - N.P.Ö., D.D.E.; Data Collection and/or Processing -N.P.Ö., D.D.E.; Analysis and/or Interpretation - N.P.Ö., D.D.E.; Literature Search - N.P.Ö., D.D.E.; Writing - N.P.Ö.

Conflict of Interest: The authors have no conflict of interest to declare.

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