Original Research Article

Graphene Oxide Nano Sheets Increase Brain Damage and Alter Dopamine, Norepinephrine and 5-Hydroxytryptamine Levels in Brain of Albino Mice

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Abstract

Background: Hazards of graphene particles, as novel application in biomedicine and industry of electronics, have attracted extensive attention. Large number of toxicological studies have evaluated the interactions of graphene nanomaterials with living systems. Although many studies have been performed on graphene-induced toxic effects, toxicological data for the effect of graphene materials on the nervous system are lacking.

Aim: To follow whether graphene oxide nano-sheets (GONs) affect malonaldehyde (MDA), glutathione (GSH) and nitric oxide (NO) contents, also, mice brain neurotransmitters levels upon using different increasing doses at different time intervals.

Materials and methods: The present study focused on the biological effects of GONs, at 10, 50, 100, 250 and 500 µg/kg bw, on mice brain content of GSH, MDA and NO after 7, 28 and 56 days of injection. Moreover, dopamine (DA), norepinephrine (NE) and 5-hydroxytryptamine (5-HT) levels, in mice brain, were also evaluated.

Results: Data obtained revealed significant decreases in GSH content coupled with significant increases in MDA and NO contents after GONs injection and these changes was dose and time dependent. Meanwhile, DA and 5- HT levels s in mice brain revealed fluctuating responses, NE level showed significant elevations at different time intervals.

Conclusion: GONs increased oxidative stress, decreased DA and increased 5-HT and NE levels in brain of adult albino mice. The neurotoxic effects were dose and time dependent.

Key words

Graphene Oxide Nano-sheets, Oxidative stress, Neurotransmitters, Brain, Mice.

Introduction

With advancements in the methods of graphene synthesis and the establishment of commercial ventures for its industrial production, the widespread use of graphene for several products has become a reality, which would lead to increased graphene environmental exposure [1]. However, the information about their potential toxicity is limited, causing potential health hazards that are similar to Carbon Nano Tubes, despite the quite different in structure and size [2].

Due to its electronic, mechanical, thermal and optical uniqueness, the two-dimensional graphene nanosheets have markedly transformed the areas of nanoscience [3]. Graphene and derivatives "miracle or wonder material", have attracted significant interest in so many technological fields due to their properties [4, 5]. These properties have led to broad-spectrum biomedical applications, such as the use in biosensors, optical imaging, drug/gene delivery, photo-thermal therapy and tissue engineering, also have been suggested for use in therapy of neurological diseases [6, 7]. As fullerenes, carbon nanotubes and graphene hold immense potential to impact several scientific disciplines, they are the most widely researched class of carbon nano-materials [8, 9]. Differing degrees of toxicity in animals or cell models may be exerted by these nano-materials, depending on the administration route or barriers they are crossing through, such as the blood brain barrier BBB [10].

Therefore, more studies are required for assessing the long-term environmental impact of graphene. As neuro-toxicological information on graphine oxide remains obscure, more research is required, because of the increase in wide applications of graphene oxide nano-materials, information should be obtained in an effort to clearly understand how neurotransmitters in the brain respond on exposure to GONs. In this study, we examined the neurotoxic effects of GONs, using five different doses, on brain content of GSH, MDA and NO as markers for oxidative stress, as well as, DA, NE and 5-HT levels after 7, 28 and 56 days of injection.

Materials and methods

Materials

All chemicals used in this investigation were of analytical grade. Graphite powder (99.9999%, Alfa Aeser, US). Potassium permanganate ($KMnO_4$, 99.9%, Merck, Germany), sulfuric acid (H_2SO_4, 98%, Merck, Germany), Sodium nitrate ($NaNO_3$, Sigma-Aldrich, St Louis, MO) and hydrogen peroxide (H_2O_2, 30%, Merck, Germany).

Preparation of Graphene Oxide Nanosheets (GONs)

According to the modified method of hummer [11, 12], GONs were synthesized from graphite powder. GONs preparation and characterization either using X-Ray Diffraction (XRD) or High Resolution Transmission Electron Microscope (HR-TEM), were carried out by [13, 14].

Experimental Design and Animal Grouping

Ninety adult male albino mice were used in this study, weighing (20-25g). Animals were provided by the National Research Center (Giza, Egypt). All mice were housed under controlled environmental conditions. Food and water were provided *ad libitum* during the designed periods. The present work was conducted according to the guidelines of the National Institute of Health for animal health and accommodation [15] and all

animal procedures were approved by the Animal Ethics Committee of Helwan University.

Mice were divided into 6 groups; first group were served as a control group, mice were intraperitoneal (i.p) injected 0.2ml normal saline solution once weekly for 8 weeks. Mice of GONs group were i.p. injected in five different doses (10, 50, 100, 250 and 500 µg/kg b.wt.) in 0.2 ml saline for 2nd, 3rd, 4th, 5th and 6th groups respectively. Animals of each group was injected once weekly for 8 weeks [16].

Brain Tissue Preparation

All mice were killed by fast decapitation at 7th, 28th and 56th days of treatment for each group. Brains were rapidly excised; wiped and dried with filter paper. Brains of each mice group were halved and stored at -80°C until use in further biochemical and neurochemical analysis.

Biochemical Investigations

The first half of brain hemisphere of each mouse was homogenized in ice-cold medium containing 50 mM Tris–HCl and 300 mM sucrose, pH 7.4 [17] and finally stored at -80°C until use in the biochemical determinations.

Determination of the Reduced Glutathione (GSH) Content

The brain GSH content of treated rats was determined for all groups [18]. The method based on the Ellman's reagent reduction with GSH to produce a yellow compound; the reduced chromogen directly proportional to GSH concentration and its absorbance can be measured at 405 nm.

Determination of Nitrite/Nitrate (NO) Level

NO content was determined in an acid medium and in the presence of nitrite the formed nitrous acid diazotise sulphanilamide which is coupled with N-(1-naphthyl) ethylenediamine. The resulting azo dye has a bright reddish purple color which can be determined at 540 nm [19].

Determination of Malondialdehyde (MDA) Content

MDA content in brain of all treated rats groups was determined According to the method described [20] by using 1ml (10%) of trichloroacetic acid and thiobarbituric acid (1ml); 0.67% and were then heated in a boiling water bath for 30 min. Thiobarbituric acid reactive substances were measured by the absorbance at 535nm.

Neurochemical Investigations

To estimate monoamines concentrations; the second hemisphere of mice brain tissue was homogenized in acidified n-butanol, and then centrifuged at 2000 r.p.m. (5 min). Thereafter; 2.5 ml of the obtained supernatant fluid were added to 1.6 ml (0.2N) of acetic acid and 5ml of n-heptane. Tubes were placed in a vortex mixer for 30 sec and centrifuged for 5 min at 1000 r.p.m. afterwards; the obtained aqueous phase was used to estimate catecholamines and indolamine levels [21].

Estimation of catecholamine Levels

Estimation of brain NE and DA levels were carried out according to [21], 1 ml of aqueous extract was used. For NE, the flourimeter excitation was adjusted to 300 nm and emission at 480 nm. While for DA, excitation was at 320 nm and emission at 375 nm by flourimeter model 6200 in Central Lab of Ain Shams University.

Estimation of Indolamine Level

The level of 5-HT was estimated by using 0.2ml of the aqueous phase [21]. Eventually; the excitation and emission were 355 and 470 nm respectively by flourimeter model 6200 in Central Lab of Ain Shams University.

Statistical Analysis

The obtained data were presented as means ± standard error. One-way ANOVA was carried out, and the statistical comparisons among the groups were performed with Post hoc test (LSD) using a statistical package program (SPSS version 14.0).

Table - 1: Effect of Intraperitoneal Injection of Graphene Oxide Nanosheets (GONs) on GSH (mg/g tissue), MDA (nmol/g tissue) and NO (µmole/g tissue) Contents in Brain of Male Albino Mice.

Days	Experimental Groups (GSH)					
	Control	**GONs 10**	**GONs 50**	**GONs 100**	**GONs250**	**GONs 500**
7	69.8 ± 3.9	53.7±3.0 (-23.1%)*	41.8±3.2 (-40.1%)*	30.0±3.7 (-57.0%)*	27.0±3.1 (-61.3%)*	16.1±1.0 (-76.9%)*
28	61.2 ± 4.4	41.1±1.3 (-32.8%)*	33.8±1.5 (-44.8%)*	27.6±2.5 (-54.9%)*	17.2±1.7 (-71.9%)*	8.4±1.0 (-86.3%)*
56	78.4 ± 2.6	65.7±5.3 (-16.2%)	56.2±2.0 (-28.3%)*	51.1±3.7 (-34.8%)*	24.5±2.4 (-68.7%)*	12.6±1.5 (-83.9%)*
	Experimental Groups (MDA)					
7	42.8±3.4	55.8±1.7 (30.4%)*	67.5±1.6 (57.7%)*	82.1±2.6 (91.8%)*	91.8±2.8 (114.5%)*	108.0±3.7 (152.3%)*
28	42.9±2.6	63.0±2.0 (46.8%)*	78.5± 2.3 (83.0%)*	97.0 ±2.0 (126.1%)*	110.1±1.7 (156.6%)*	116.1±4.5 (170.6%)*
56	56.1±1.9	81.6±3.4 (45.4%)*	101.3±2.8 (80.6%)*	117.5±1.8 (109.4%)*	133.2±2.5 (137.4%)*	148.7±2.8 (165.1%)*
	Experimental Groups (NO)					
7	112.3±2.9	133.9±1.9 (19.2%)*	152.2±2.5 (35.5%)*	177.5±3.3 (58.0%)*	240.1±5.9 (113.8%)*	340.5±2.3 (203.2%)*
28	99.5±5.0	127.5±3.3 (28.1%)*	144.4±2.8 (45.1%)*	185.9±6.1 (86.8%)*	242.5±5.0 (143.7%)*	411.8±5.5 (313.9%)*
56	96.7±4.9	108.9±2.7 (12.6%)	154.8±8.3 (60.1%)*	270.5±4.2 (179.7%)*	366.2±4.1 (278.7%)*	459.5±4.7 (375.2%)*

The number of animals was 5 in each treated group.

Data are expressed as mean ± SEM. * Significant change at $p<0.05$ as compared to control group.

( ) % difference with respect to control value.

Results

Effect of GONs on GSH, MDA and NO as Oxidative Stress Markers in Mice Brain Tissue Homogenate

The present study demonstrated that (i.p) injection of GONs to adult albino mice induced marked and significant effects on the oxidative stress markers of the mice brain. The data expressed from **Table - 1** indicated that all GONs injected doses of (10, 50, 100, 250 and 500 µg/kg bw) provoked a marked, gradual and sharp significant reduction (at $p < 0.05$) of GSH content in mice brain with a percentage values of (-23.1%, -40.1%,-57.0%,-61.3% and -76.9%) at the 7th day; also, at the 28th day (-32.8%, -44.8%, -54.9%, -71.9% and -86.3%) and at the 56th day of GONs injection(-16.2%, -28.3%, -34.8%, -68.7% and -83.9%), respectively. As obviously presented, GONs at a dose of 500 µg/ kg bw , induced the maximum reduction in GSH content of mice brain at 7, 28 and 56 days of injection with values of (-76.9%, -86.3% and -83.9%), respectively, when compared with their respective control groups. Furthermore, injection of GONs, at a dose of 10 and 50 µg/kg bw, for 56 days revealed gradual recovery, though not reaching the control value of GSH content in mice brain, indicating the ability of mice brain to recover its GSH content.

Data elucidated in **Table - 1** concerning the MDA content of mice brain after injection of GONs at doses (10, 50, 100, 250 and 500 µg/kg bw), revealed a gradual dramatic sustained marked increase in MDA and NO content of a significant change (at $p < 0.05$) with increasing the dose, if compared with their control values.

The increase in MDA and NO contents for 10, 50, 100, 250 and 500 µg/kg bw, treated groups, were at the 7th day with percentage values of (30.4%, 57.7%, 91.8%, 114.5% and 152.3%) and (19.2%, 35.5%, 58.0% 113.8% and 203.2%),respectively, and at the 28th day with values of (46.8%, 83.0%, 126.1%, 156.6% and 170.6%) and (28.1%, 45.1%, 86.8%, 143.7% and 313.9%) for MDA and NO contents , respectively, when compared with their corresponding control values. Though showing lesser effect than the 28th day groups, MDA content of mice brain homogenate remained elevated after 56 days, for all GONs doses, with a significant change (at $p < 0.05$).

GONs at a dose of 10 µg/kg bw, showed an increase of a non-significant change in animals brain content of NO after 56 days of GONs injection indicating the ability of mice brain to adapt and restore NO content to reach about the control values. Meanwhile, NO content of mice brain indicated a tremendous increase with a significant change (at $p < 0.05$), with percentage difference of (60.1%, 179.7%, 278.7% and 375.2%) at a dose of (50, 100, 250 & 500 µg), respectively, if compared with their control values. The present results indicate increasing severity of brain damage by continuous increasing doses of GONs. The present data evaluating the effect of repeated increased doses of GONs on mice brain for 7, 28 and 56 days indicated that the effect of GONs was dose and time dependent.

Effect of GONs on DA, NE and 5-HT in Mice Brain

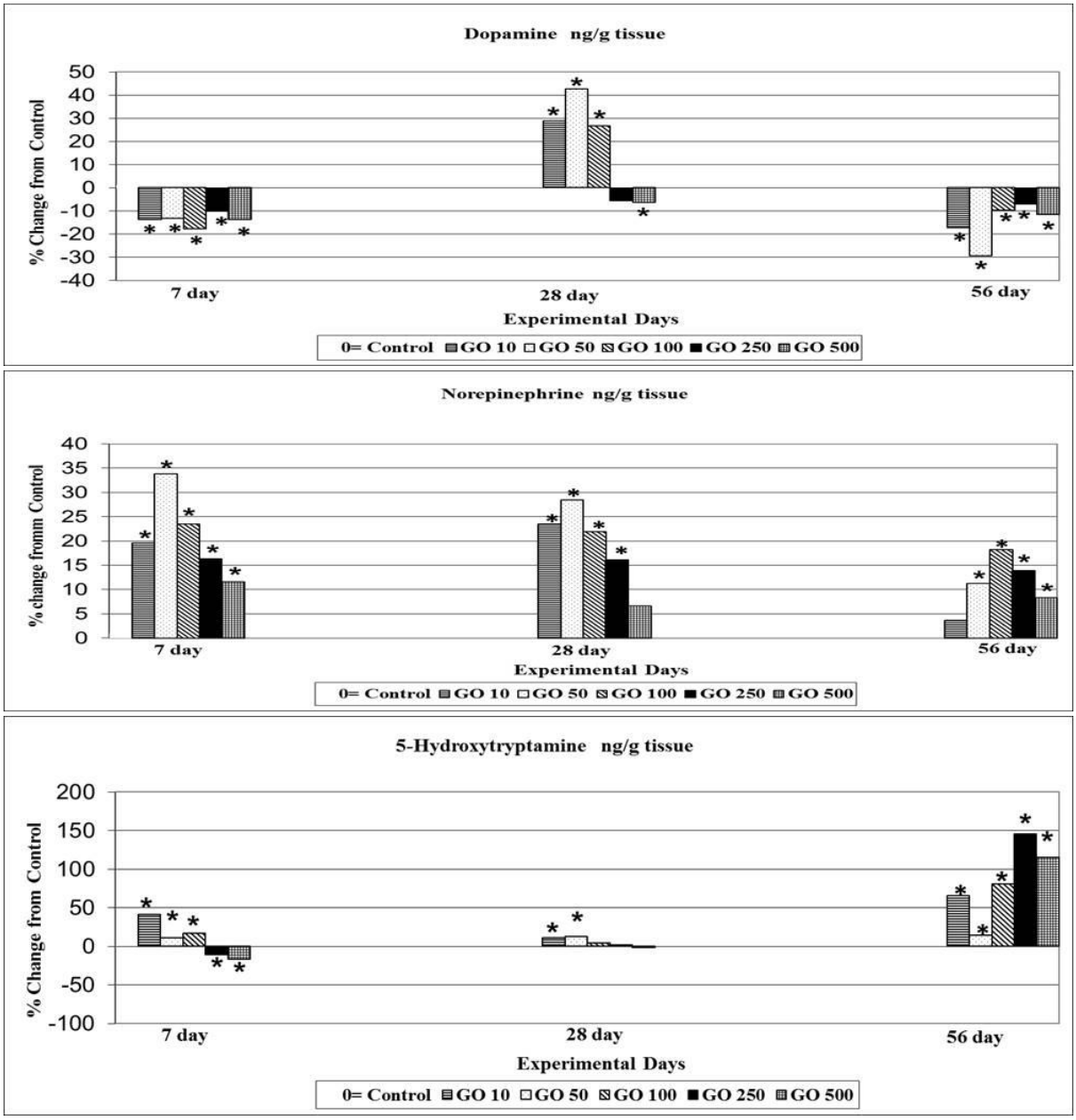
The effect of i.p. injection of GONs (10, 50, 100, 250 and 500 µg/kg, bw) on DA, NE and 5-HT levels in brain of male albino mice after 7, 28 and 56 days of treatment were illustrated in Figure (1). Results of the present study revealed that injection of GONs (10, 50, 100, 250 and 500 µg/kg bw) decreased DA level, at the 7th and 56th days of treatment, with a significant change (at $p < 0.05$) with percentage difference of (-13.8%,-13.2%,-17.8%,-10.2% and-13.7%) and (-17.2%, -29.4%, -9.6%, -6.9% and -11.6%), respectively, compared with control values. Meanwhile, injection of GONs at 10, 50 & 100 µg/kg bw increased, with a significant change (at $p < 0.05$), DA level in mice brain homogenate at 28th day of treatment, the observed increase was of percentage difference (28.9%, 42.7% and 26.6%), respectively, if compared to control values. At the same day of treatment, GONs in both doses, 250 and 500 µg/kg bw, induced a decrease in DA level of mice brain. The dose of GONs at 500 µg/kg bw, was more effective in lowering the DA level, being of a significant change (at $p < 0.05$), with percentage difference of (-6.4%) with respect to control value.

Data concerning the NE level of adult mice brain, revealed that GONs injection increased its level with a significant change (at $p < 0.05$) in all treated groups, injected with GONs at all doses (10, 50, 100, 250 and 500 µg/kg, bw), and the highest percentage difference was recorded after injection of 50 µg/kg bw, at the 7th and 28th days being of 33.9% and 28.5%, respectively, if compared to control values. NE level in adult mice brain tended to approach normal control values, recording an increase with a non-significant change after 56 days and 28 days of 10 and 500 µg/kg bw injection with GONs.

Exploring the effect of GONs injection on 5-HT level in mice brain homogenate, 5-HT level was found to increase, with a significant change (at $p < 0.05$) after 7 and days of treatment with GONs at (10, 50 and 100 µg/kg, bw), and the 10 µg dose recorded the highest percentage difference (40.6%), when compared to control values. Meanwhile, GONs at doses 250 and 500 µg/kg bw, recorded a decrease of a significant change (at $p < 0.05$) at the same day of treatment of (-10.4% and -16.8%), respectively, if compared to control values. By continuous injection of different doses, 5-HT level was found to increase significantly, at 56 day with the highest percentage difference at 10, 100, 250 & 500 µg GONs/kg bw, with 65.7%, 80.6%, 145.8% and 115.0%, respectively, if compared to control values (**Figure – 1**).

Figure - 1: Effect of Intraperitoneal Injection of Graphene Oxide Nanosheets (GONs) (10, 50, 100, 250 and 500 µg/kg, bw) on DA, NE and 5-HT Levels in Brain (ng/g tissue) of Male Albino Mice after 7, 28 and 56 Days of Treatment.

* Significant change at $p<0.05$ as compared to control group



Discussion

Toxicological studies *in vitro* and *in vivo* have evaluated the interaction of graphene-based nanomaterials with living systems ranging from prokaryotes to eukaryotes; as microbes, mammalian cells, and animal models depending on dosage and functionalization with various reducing and stabilizing agents [22]. Short- and long-term *in vivo* toxicity studies and bio-distribution of synthesized graphene have been examined by variety of methods and starting materials [1].

Graphene possesses attractive physical and chemical properties due to its unique structure: exceptionally high electron mobility, thermal conductivity, optical transmittance, mechanical strength, chemical stability, and surface area-to-volume ratio [4, 8]. Graphene oxide (GO), which is chemically exfoliated from oxidized graphite,

has facilitated its applications in the biomedical fields and biological applications being used as delivery carriers, biosensors, and in gene therapy [23, 24]. As documented previously [13, 14], the d-spacing increase of GONs, used in the present study using XRD analysis was attributed to the presence of abundant oxygen-containing functional groups on both sides of the graphene sheets caused by oxidation proposing large exfoliation of the layered GONs as confirmed earlier [25].

Moreover, the toxicological impact of graphene and GONs were described [26], as they found that small size of nanoparticles facilitates their uptake into cells and also, transcytosis across epithelial cells into blood and lymph circulation [27]. Being in close contact with the cell or tissue surface, graphene therefore, maximizes its utility in bio-applications, raising the concern of unexpected biological consequences [28, 29].

It is critical to evaluate GONs potential neurotoxicity on MDA, GSH and NO content, also, DA, NE and 5-HT levels in brain of adult mice, therefore, five doses of GONs were selected to investigate its possible toxicity. Neurons and glial cells are vulnerable to oxidative damage due to their high content of unsaturated fatty acids, elevated oxygen consumption rate, and relative limited content of antioxidant enzymes compared with other organs [30].

In the present study, MDA (marker of oxidative stress), GSH (an important antioxidant molecule that prevents cellular damage induced by ROS), and NO contents (a signaling molecule that plays a key role in inflammation pathogenesis and contributes in regulation of apoptosis, also, a potent neurotransmitter at the neuron synapses) [31], were evaluated to estimate the changes accompanying GONs injection. Results obtained, revealed a dose dependent gradual significant sustained decrease in GSH content accompanied by sustained significant increase in MDA and NO contents of GONs treated mice group indicating the neurotoxic effects of GONs on mice brain. Oxidative stress reaction has been identified as the common mechanism by which GONs exert their cellular neurotoxicity [32, 33].

Nanomaterials have the potential to cause organ damage throughout the body as they have shown to enter systemic circulation [34]. Moreover, in *in vivo* study, a severe toxic damage of GONs was observed and localized in the lung, liver, and spleen, after intravenous administration [16]. The BBB has a complicated physical and molecular structure providing suitable microenvironment for neuronal activity to withstand the penetration of foreign substances, including graphene materials. Graphene materials are believed to translocate into the brain through the BBB, due to their tiny size [35]. Moreover, intraperitoneal injection of GON particles in male mice for seven days caused brain damage, because of their unique physicochemical properties [36]. Oxidative stress is believed to be a major toxic response to GONs at toxic doses, and the generation of reactive oxygen species (ROS) is considered its main factor [37-39].

Oxidative stress may result from inflammation which was evident, in the present study, by the dose dependent gradual significant increase in NO brain content of GONs treated mice, having consequent damaging effects on DNA and proteins, involved in brain damage as previously documented [40]. Graphene toxicity was associated with generation of intracellular ROS, due to its passive internalization into cells (endocytosis) [41], active internalization [42], or actin-dependent macropinocytosis [43] thereby causing damage to proteins and DNA leading to cell death via apoptotic or necrotic pathways [41]. GSH depletion is one of the important clues in cellular toxicity [36]. Hydrogen peroxide was found to activate the transcription factor, NF-κB involved in cellular apoptosis, therefore, playing a crucial role in many neurodegenerative diseases [36]. Neurotoxicity was proposed through lipid peroxidation of the membrane, following excessive generation of ROS induced by GO, and subsequent overwhelming of antioxidant defenses of cells.

So, Graphene mediated ROS damage were elucidated by two mechanisms; first; interference with the electron transport system after cellular internalization, and overproduction of H_2O_2 and OH^-, thereby, triggering the release of cytochrome c activating caspase 9, 3 and 7complex (cyt c) and finally, cell death [44]. Second; induction of the mitogen activated phosphokinase MAPK and TGF- β signaling pathways leading to activation of Bcl-2 proteins followed by activation of mitochondria-induced apoptosis [45]. In addition to ROS-induced cell death, activation of toll-like receptors [46], inducing proinflammatory pathways involved NF-κB leading to production and secretion of cytokines and also, chemokines. Moreover, systemic delivery of GO caused significant production of IL-6 and TNF- α in fluids and serum of mice [41], activating cell surface toll-like receptors, inducing autophagy via the inflammatory pathways [46]. As a consequence of GONs accumulation, the granulomatous reaction as an inflammatory response was commonly found among the internal organs [47].

The present study, indicated altered levels of DA and 5-HT, especially, after 7 and 56 days of treatment for all doses used and at a dose of 250 and 500 μg/kg body weight at 28 days of treatment. Lung apoptosis was observed in mouse after GONs inhalation, as a major toxic response [48]. Some apoptosis-related proteins were activated following GO exposure in vitro [45, 48]. Furthermore, GONs treatment was found to inhibit neural PC12 cell lines proliferation and induced high levels of apoptosis in a dose-dependent manner [49]. The toxicity differences of graphene-based nanomaterials were attributed to their physicochemical properties, such as density of functional groups, size, and conductivity [50].

Potent toxic effects due to GONs exposure, were manifested by reduced cell viability, increased lactate dehydrogenase release, mitochondrial dysfunction and ROS generation [23, 37]. Cell cycle alteration and apoptosis were found to be related in many cases [51]. Nanomaterials may lead to arresting of cell cycle at various phases as documented earlier [52-54]. GONs were found to interact with the cytoskeletal components of the cell, by adhering and wrapping around the cell membrane, inserting into the lipid bilayer thus perturbing membrane integrity and inducing cell-cycle alterations, apoptosis, and oxidative stress and become internalized into cells [55,56].

Moreover, GO was found to induce cell cycle arrest at the G0/G1 phase [57-59] which was attributed to the location of GO on F-actin filaments [57] relating to abnormal growth and altered capacity for mitotic division. GO caused apoptosis and cell cycle arrest which were the main toxicity responses to GO treatment [49]. Changes in extracellular signal-related kinases (ERK) signaling pathway molecules (important protein kinase of the cascade which control numerous cellular processes, including proliferation, differentiation, development, stress response, and apoptosis) after exposure to 50 μg/mL GO and reduced GO were documented [49]. Therefore, the unregulated cell cycle and apoptosis [60, 49] may explain the altered levels of DA and 5-HT in the present results.

Recent studies have documented that neuronal tissues of brain of GONs displayed, microscopically, abnormal changes, so that, some of the neuronal cells of both cerebral and cerebellar cortices showed degeneration and necrosis, being more noticeable in 500 mg/kg GONs treated animals than in animals receiving 150 or 50 mg/kg GONs [47]. They also, indicated the distortion of cerebellar layers of all GONs treated animals, accompanied by varying degrees of degeneration and necrosis and necrotized Purkinje cells and were most conspicuous and more severe in rats receiving 500 mg/kg GONs [47], which may agree with results of the present work indicating the severe effect of GONs at dose of 500 μg/kg on neurotransmitters levels.

Furthermore, after being internalized, graphene was found to induce DNA cleavage due to interactions; such as hydrophobicity, and

electrostatic interactions [61, 62]. The surface charge distribution on graphene sheets was found to play an important role in the activation of kinases and release of calcium, eventually leading to platelet aggregation [63]. In the present study GONs injection caused potent toxic responses, as nanomaterials trigger specific biochemical and biological responses and that these toxic effects are caused by the generation of ROS, including apoptosis [64].

The toxicological mechanisms of GONs revealed, involvement of the inflammatory response, oxidative stress, DNA damage, apoptosis, autophagy, and necrosis [65, 66]. Quantitative proteomics proposed interference of GO with various pathways in metabolism and cell energy production [67], causing mitochondrial damage, including a decrease in its membrane potential, reduction of ATP production, dys-regulation of Ca^{2+} homeostasis, interference with electron transport chain through disturbance of the electron transfer, and overproduction of ROS [68].

Furthermore, cholesterol, essential for synaptic vesicle origination, distribution, and turnover [69] being a key constituent of membrane nanodomains (lipid rafts), acting as the nexus for transmembrane protein complexes, mediating signal transduction across the plasma membrane, and carrying out receptor-mediated endocytosis [70]. Recently, *in vitro* studies, synaptic vesicle changes in presynaptic terminals on graphene substrates leading to increased releasable vesicles, by ~30% increase in total dye loss in neurons on graphene, indicating an increased pool of releasable vesicles ,showing increased probability in their release which are stimulated by graphene substrates, leading to potentiation of neurotransmission due to neurotransmitter release [29].

The present results concerning NE level in brain of GONs treated mice show biocompatibility of GONs with NE releasing neuronal cells; referring to the ability of GONs to interact with cells, tissues, or the body without causing harmful effects [22]. Graphene and GO were found to cause simultaneous differentiation in culture of mouse induced pluripotent stem cells (iPSCs), as they exhibited normal cell adhesion and proliferation a graphene surface, whereas iPSCs cultured on a GO surface were found to adhere and proliferate at a faster rate [71]. Furthermore, GO was found to effectively promote dopamine neuron differentiation and enhance dopamine neuron-related gene expression [72], compared with untreated cells, which explain the observed increase in DA, after 28 days, and 5-HT levels after 7 and 56 days of treatment. NE levels increased at 7, 28 and 56 days suggesting that NE level in tissue may reflect biocompatibility of GONs with mice brain tissue.

The toxicity or biocompatibility depends on the functionalization of graphene, which can reduce its toxic effects. Growth of hippocampal neurons, *in vitro* induced rat embryonic hippocampal neuronal neurite outgrowth and adhesion [73]. Carbon Nano Tubes can lead to formation of selected networks on different matrices due to neuron growth, proliferation, and adsorption of molecules on their surface. Hippocampal neurons were found to increase their synaptic activity by substrate increment as the single-cell patch-clamp technique recorded spontaneous postsynaptic currents, but remaining with the same action potential [74]. Furthermore, earlier reports showed that the crystallinity of graphene promotes cell adhesion and neurite outgrowth of mouse neurons grown on bare graphene, suggesting the significance of close contact between graphene and the cell surface in mediating graphene's effects [75]. Aggregated graphene used as a substrate or applied acutely to mature neuronal cultures resulted in few developmental or morphological changes [76, 77].

Conclusion

The present study indicated that GONs administration at doses of 10, 50, 100, 250 and 500 μg/kg bw caused neurotoxicity indicated by a significant sustained decrease in GSH content

with significant sustained increases in MDA and NO contents accompanied with significant decreases in DA and 5-HT levels at different time intervals reflecting the toxic effect of GONs through increasing oxidative stress, inflammation, production of ROS and degeneration of mice neuronal brain cells which affected the release of DA from DA-ergric releasing neurons. Meanwhile, NE and 5-HT releasing neurons showed significant increases after 7, 28 and 56 days of treatment, especially for NE, indicating ability of GONs bio-compatibility in their releasing neurons helping in enhancing their release.

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