Colloids and Surfaces B: Biointerfaces 157 (2017) 335–346

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Original Research Article

Developmental neurotoxic effects of graphene oxide exposure in zebrafish larvae (*Danio rerio*)

JC Soares [a], TCB Pereira [a], KM Costa [b], T Maraschin [c], NR Basso [c], MR Bogo [a,b,d,*]

[a] *Programa de Pós-Graduação em Biologia Celular e Molecular, Laboratório de Biologia Genômica e Molecular, Faculdade Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Brazil*
[b] *Programa de Pós-Graduação em Medicina e Ciências da Saúde, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Brazil*
[c] *Programa de Pós-Graduação em Engenharia e Tecnologia de Materiais, Faculdade de Química, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Brazil*
[d] *Instituto de Toxicologia e Farmacologia, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Brazil*

ARTICLE INFO

Article history:
Received 21 February 2017
Received in revised form 11 May 2017
Accepted 30 May 2017
Available online 3 June 2017

Keywords:
Graphene oxide
Nanotoxicology
Neurotoxicology
Autophagosome
Zebrafish larvae

ABSTRACT

Although graphene oxide (GO), a nanomaterial with hexagonal planar layer, has been widely studied due to its applications in neurobiology that include drug delivery and tissue engineering, additional studies to assess its potential toxic effects are still needed. Thus, this study evaluated the effects of GO exposure (at 5, 10, 50 or 100 mg/L) during six consecutive days on mortality, hatching, spontaneous movement, heart rate, morphology, locomotion behavior, acetylcholinesterase (AChE) activity, dopamine levels and relative gene expression of developmental neurology-related genes using zebrafish larvae. In the 5 mg/L dose, *synapsin IIa* expression up-regulation was seen concomitantly with down-regulation of *dat* expression, showing a potential compensatory mechanism. Moreover, the 10 mg/L exposure caused an increase in heart rate, in absolute turn angle, brain cell damage and a decrease in dopamine levels. These alterations may be associated with autophagosome formation found in GO-exposed larval brain. No changes were observed on higher doses of GO exposure, probably due to nanomaterial agglomeration. Taken together, these results show that toxic effects of GO exposure are not dose-dependent, and are preeminent in lower concentrations. Additional studies are needed to deepen the specific mechanisms of GO neurotoxicity and are required to elucidate its potential biomedical use.

1. Introduction

Graphene oxide (GO) is a nanomaterial characterized by sp^2-hybridized carbon atoms with three in-plane σ-bonds per atom, forming a hexagonal planar layer in a honeycomb-like atomic arrangement [1]. Due to extraordinary properties, such as large specific surface, conductivity, flexibility and quantum properties, GO have been studied in the biomedical field as a potential nanocarrier for drugs. Incorporation of graphene-based nanoparticles to enhance *in vivo* visualization of brain tumors [2] and anti-cancer drugs loading [3] are examples of applications. Additionally, GO surfaces have been studied to enhance neurite sprouting and outgrowth [4], as neural interface in tissue engineering [5] and as platform for generation of functional neuron-like cells from human neural stem cells [6]. Due to increasing interest in GO applications in several fields including neurology, toxicological evaluations are important to clarify GO potential application in medicine. Studies in nanomaterials field have reported evaluation of some carbon-based nanoparticles neurotoxicity *in vitro* and *in vivo*. Previous study [7] have found neurotoxic effects of multi-walled chemically functionalized carbon nanotubes (MWNTs) exposure in neuronal and glial cells culture derived from rat frontal cortex (FCO) and striatum (ST), respectively. Glial cells were found to be more sensitive in ST than FCO group, showing brain region-specific toxic effect. A second study, using an intraperitoneal administration of the same nanomaterial, MWNTs, in rats demonstrated cognitive abilities deficit, impaired motor function, decreased synaptic plasticity by abnormally enhanced depotentiation and decreased excitatory postsynaptic potential in hippocampus [8]. Nanodiamond, another promissor carbon nanomaterial for medical applications, was previously known to not induce citotoxicity in mouse dorsal root ganglion neurons (DRG) and hippocampal neurons, even though, a dose-dependent decrease of neurite length and a drastic neurite morphology alteration was observed in DRG [9]. Further, fullerenol effects have been evaluated in rat hippocampal neu-

* Corresponding author at: Programa de Pós-Graduação em Biologia Celular e Molecular, Laboratório de Biologia Genômica e Molecular, Faculdade Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Av. Ipiranga, 6681, Prédio 12. CEP: 90.619-900, Porto Alegre, Brazil.
E-mail address: mbogo@pucrs.br (M. Bogo).

http://dx.doi.org/10.1016/j.colsurfb.2017.05.078
0927-7765/

ronal culture exposed to lead, demonstrating protective effects in low concentrations in terms of viability improvement, although, high concentrations decreased the viability and induced neurons apoptosis [10]. Regarding toxicological studies with GO in neurology field, it has been reported that exposure to high doses of GO nanoribbons in neuroblastoma cell culture, induce reactive oxygen species (ROS) generation, mitochondrial swelling, autophagosomes formation and higher rate of necrosis [11]. Chen *et al.* [12] demonstrated *in vivo* that this nanomaterial may translocate from the environment to the brain of zebrafish larvae, causing mitochondrial damages, ROS generation and DNA methylation. However, few is known about the potential specific toxic mechanisms of GO in nervous system (NS) *in vivo,* thus, more in-depth nano/neurotoxicology studies are necessary to evaluate the effects of graphene oxide in whole-animal approach, especially because stability, concentration and time exposure of nanoparticle suspensions may result in different outcomes.

Zebrafish (*Danio rerio*) has been recognized as popular animal model due to exceptional set of characteristics, such as high similarity to human genome and gene function [13], small size, high reproducibility, fast development and transparency of embryo and larvae, which allows cells and organs observation since early stages [14]. Zebrafish has been widely used in nanotoxicology field to evaluate effects of nanomaterials as such silver, gold, carbon nanotubes and fullerenol in the nervous system [15–17]. Among various parameters assayed in previous neurotoxicology studies, the dopaminergic system has been widely evaluated, since it participate controlling numerous functions in the brain including motor activity [18]. This system works through dopamine (DA) modulation in the brain, that is re-uptaked by dopamine transporter (DAT) into presynaptic neuron [19]. Toxic effects in this system have been associated with alterations in behavior. Indeed, Bortolotto *et al.* [20] have associated absolute turn angle alterations with parkinson-associated symptoms in adult zebrafish exposed to paraquat. Regarding GO, previous studies showed that GO exposure resulted in loss of dopaminergic neurons in diencephalon region of zebrafish larvae resulting in reduction of spontaneous movements [21]. More recently, the effect of GO exposure in parental zebrafish was evaluated in their offspring, and neurotoxic effects including loss of dopaminergic neurons, autophagy, ubiquitin decrease and β-galactosidase increased in larvae were reported [22], corroborating previous GO neurotoxic-associated findings [21]. Additionally, dopaminergic neurons in zebrafish differentiate around 18 hpf (hours post fertilization) [23] showing potential as model to investigate toxic effects and diseases associated with this neurosystem, such as Parkinson's disease, in early life-stages [21].

Taking into account (1) the increase of GO potential applications in biomedical field, (2) the potential neurotoxic effects and mechanisms associated with GO administration and (3) zebrafish consolidated use as animal model in nanotoxicology and neurotoxicology, in this study, we focused on analyzing the effects of GO exposure during six consecutive days (embryogenesis and larval stages) on several parameters including (i) mortality, hatching, spontaneous movement, heart rate, morphology and behavior locomotion, as well as in (ii) gene expression of neurotoxicity-related markers (iii) AChE activity and dopamine levels in whole larvae and (iv) analyses of brain cells ultrastructure by transmission electron microscopy (TEM).

2. Materials and methods

2.1. Preparation and characterization of GO

GO was synthesized from GRAFINE 99200 (Nacional de Grafite Ltda., Brazil) with ≈ 75 μm, using a modified Staudenmaier method [24]. The oxidation reaction lasted 24 h and then graphite oxide was exfoliated via ultrasonic vibration during 4 h to produce GO. At the end of the reaction, aqueous HCl solution (10% v/v) was added to the suspension to remove sulfate ions. Deionized water washes followed by centrifugation were performed several times in order to reach pH 3. GO was then maintained within a dialysis membrane in water until pH 6 was achieved. The suspension was dried at 150 °C resulting in a thin film.

GO *x-ray diffraction* (XRD) measurements were performed on a diffractometer (Rigaku, DMAX 2200) equipped with a Cu tube (λ = 0.15418 nm) and a secondary monochromator. The goniometer Siemens D500 with scintillator (NaI and Tl) as detector was employed. XRD samples were prepared by coating the sample holder with a uniform layer of powdered samples at room temperature. From XRD parameters, the interlayer distances (d002) in the graphene and the number of graphene layers were estimated using Bragg's Law and the Scherrer equation according to Pavoski et al. [25].

Raman analyses were performed at room temperature using a microscope (Olympus) and an iH320 JobinYvon Spectrometer with a CCD (charged coupled device) detector, cooled by liquid nitrogen. The excitation source was a 10 mWHeNe laser (632.8 nm) and the acquisition time was 20s.

The GO surface image was obtained through *Atomic Force Scanning Microscopy* (AFM) using a Dimension Icon PT microscope from the Bruker. The samples were suspended in distilled water and sonicated for 4 h. Three drops of the resulting suspension were deposited on ultrafiltration substrate and oven-dried at 100 °C for 10 min. The graphene layers stacks size (nm) were obtained by *Nano Scope Software* performing 20 measurements (n = 20) for greater reliability. The samples morphological evaluation was obtained with *Field Emission Scanning Electron Microscopy* (SEM-FEG), using a FEI Inspect F50 microscope, operating at 20 kV. The sample was prepared through deposition of the powdered material on an aluminum stub and metallized with gold.

2.2. Preparation and characterization of GO suspensions

GO suspensions at 5, 10, 50 and 100 mg/L [26], were daily prepared by dispersion of GO dry sheets in 1% Pluronic F 68 (PF68) water solution (GOPF). Water of zebrafish re-circulating system (Tecniplast) was used to prepare the suspensions, which were sonicated for 1 h in ultrasonic bath at 40 kHz/60W, and then agitated overnight. Additionally, two GO-free controls, with and without 1% PF68 solution were prepared and submitted to same process. Conductivity and pH of each solution were monitored daily, before exposure, and maintained at 300–1500 $\mu S/cm^{-1}$ and 6.8–8.5, respectively [27].

To evaluate GO suspensions stability, absorbance at 230 nm was monitored automatically in each concentration every two hours, during 24 h, through UV–vis spectroscopy (Hewlett Packard 8453, HP) [28]. Furthermore, GOPF suspensions were spontaneously dried at room temperature and analyzed by *Transmission Electronic Microscopy* (TEM) (Tecnai G2 T20, FEI) to qualitatively evaluate GO agglomerates morphology in PF68/water suspension. To confirm GO agglomerate formation and to measure its size in suspension, *Dynamic Light Scattering* (DLS) analyses were performed with a Zetasizer (ZEN3600, Malvern).

2.3. Fish maintenance and exposure

Adult wild-type zebrafish stocks were housed in automated re-circulating system (Tecniplast) using default conditions of temperature (28° ± 2 °C), light/dark cycle (14:10 h), and pH (6.8–8.5) [27]. Fish were fed three times a day using dry flake food (TetraMin Tropical Flake Fish®) supplemented with brine shrimp (*Arthemia*)