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Interactions of oxidized multiwalled carbon nanotube with cadmium on zebrafish cell line: The influence of two co-exposure protocols on *in vitro* toxicity tests

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ABSTRACT

The widespread production and application of carbon nanotubes (CNT) have raising concerns about their release into the environment and, the joint toxicity of CNT with pre-existing contaminants needs to be assessed. This is the first study that investigated the co-exposure of oxidized multiwalled carbon nanotubes (ox-MWCNT) and cadmium (Cd) using a zebrafish liver cell line (ZFL). Two *in vitro* co-exposure protocols differing by the order of ox-MWCNT interaction with Cd and fetal bovine serum (FBS) proteins were evaluated. Ox-MWCNT was physical and chemical characterized and its adsorption capacity and colloidal stability in cell culture medium was determined in both protocols. Cytotoxicity was investigated by MTT, neutral red, trypan blue, lactate dehydrogenase assays and the necrosis and apoptosis events were determined using flow cytometer. The Cd presence in medium did not interfere in the protein corona composition of MWCNT but the order of interaction of FBS and Cd interfered in its colloidal stability and metal adsorption rate. The ox-MWCNT increased Cd toxicity at low concentration probably by a "Trojan horse" and/or synergistic effect, and induced apoptosis and necrosis in ZFL cells. Although it was not observed differences of toxicity between protocols, the interaction of ox-MWCNT first with Cd led to its precipitation in cell culture medium and, as a consequence, to a possible false viability result by neutral red assay. Taken together, it was evident that the order of compounds interactions disturbs the colloidal stability and affects the *in vitro* toxicological assays. Considering that Protocol A showed more ox-MWCNT stability after interaction with Cd, this protocol is recommended to be adopted in future studies.

1. Introduction

The industrial production of nanomaterials (NMs) is increasing exponentially, however, the knowledge about the effects and potential risks of NMs to the environment and aquatic biota are still incipient (Bennett et al., 2013; Ferreira et al., 2014; Sanchís et al., 2016). The ecotoxicological effects of NMs depend on their physical and chemical characteristics, such as size, shape, surface area, diffusion capacity, aggregation/agglomeration properties in suspension, functionalization as well as their interactions with surrounding environments (Handy et al., 2012; He et al., 2014; Wang et al., 2016a).

Nanomaterials can potentially interact with contaminants in the environment and affect their distribution, accumulation, and toxicity (Deng et al., 2017). However, in the literature is rare detailed information about how the co-exposures studies were conducted in order to improve the reproducibility of data. Then, exposure standardizations of NMs still needed, especially in joint toxicity studies with nanomaterials and other contaminants (Hussain et al., 2015; Song et al., 2017; Wang et al., 2015).

Carbon nanotubes (CNTs) have numerous applications in industrial, agriculture and biomedical sectors (Freixa et al., 2018; Smajda et al., 2010; Umbuzeiro et al., 2011). Previous studies have already predicted relevant concentrations of these NMs in the environment in the future years due to increasing application demand or accidental circumstances

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(Gottschalk et al., 2013; Keller and Lazareva, 2014). In these studies, the predicted concentrations for CNTs in effluents were 0.005–0.05 μg L^{-1} and 0.05–5 mg kg^{-1} in biosolids by Keller and Lazareva (2014) and 0.05–0.1 μg L^{-1} by Gottschalk et al. (2013).

In the aquatic environment, CNTs may interact with metals and act as carriers of them, facilitating their entry into cells and potentiating their toxicity (Costa et al., 2012; Ferreira et al., 2014; Henry et al., 2011; Limbach and Wick, 2007). For example, multiwalled CNTs (MWCNTs) enhanced the acute toxicity of Pb and pesticides on the freshwater fish *Oreochromis niloticus* (Campos-Garcia et al., 2015; Martinez et al., 2013), the toxicity of Cd (Wang et al., 2016a) and AsO_3^{3-}, AsO_4^{3-} in *D. magna* (Wang et al., 2016b) and HepG2 cells (Yu et al., 2016). MWCNT also enhanced the photosynthesis inhibition effect of the Diuron herbicide (Schwab et al., 2013) and increased the mobility inhibition effect of phenanthrene (Zindler et al., 2016). Furthermore, treated CNTs improve their water solubility and dispersion in biological media, and acid-treated MWCNTs (ox-MWCNTs) are efficient at removing pollutants, such as Cd and other metals from water (Bhanjana et al., 2017; Ravi and Vadukumpully, 2016). Thus, the potential risks associated with the joint toxicity of heavy metals and NMs needs to be evaluated.

Among the metals, Cd is a non-essential and toxic heavy metal that has been found in large quantities in the environment as a result of industrial activities and the use of fossil fuels (Balmuri et al., 2017; Chen et al., 2014). In aquatic environments, Cd can accumulate into biota and cause harmful effects to them as well as to humans, via food chain (Han et al., 2009; Olsvik et al., 2016). Cd toxicity is based on ionic mimicry, as it replaces other elements, e.g. calcium and zinc (Sandbichler and Höckner, 2016), and it accumulates primarily in the liver and kidneys, causing kidney and bone dysfunction and inducing carcinogenesis by numerous mechanisms (Liu et al., 2009; Zhang et al., 2014).

In vitro methodologies are widely used to assess the environmental effects of complex mixtures of pollutants (Stadnicka-Michalak et al., 2014), evaluating the uptake mechanisms and cell sensibility to chemical and physical agents (Bonomo et al., 2016; Raffa et al., 2010). However, to evaluate NM effects *in vitro*, mainly of CNTs, it is important to take some precautions. In the presence of serum proteins, NM surfaces are covered by a "protein corona (PC)" formed by multiple proteins that may influence the NM toxicity that can modify membrane interactions, accumulation, mechanism of endocytosis and stress reactions (Hussain et al., 2015; Sharma et al., 2014; Winzen et al., 2015). Therefore, the PC becomes the interface between NMs and cells, and its analysis is essential to understand its role in toxicity studies *in vitro*.

Zebrafish (*Danio rerio*) is an excellent model for toxicity studies due to biological similarities with humans (Costa et al., 2012; Eide et al., 2014). Humans and zebrafish genome sequences have demonstrated conservation in cell cycle genes, tumor suppressors and oncogenes (Shive, 2013); similarly, zebrafish cancer is histologically and genetically similar to human cancers. In addition, as liver is highly susceptible organ to Cd, the toxicity and accumulation of this metal in zebrafish liver (ZFL) have been reported in cells cultured *in vitro* (Chen et al., 2014; Tang et al., 2013).

It is evident that CNTs are important potential pollutants and Cd is considered one of the most toxic heavy metals for aquatic biota (Ji et al., 2016). Both contaminants are associated with a wide variety of applications in electronic engineering, photovoltaic devices, automotive industry (Chen et al., 2014; Freixa et al., 2018). In this context, this study evaluated the effects of the interaction of ox-MWCNTs with Cd in a zebrafish liver cell line (ZFL). Additionally, we aimed to evidence the influence of the order of carbon nanotube and metal interaction protocols towards co-exposure toxicity studies as the interaction order possible produce different complexes having different stabilities, and then, different toxicity effects.

2. Materials and methods

2.1. Preparation and characterization of oxidized multiwalled carbon nanotubes

Industrial grade multiwalled carbon nanotubes (MWCNT) were obtained from CNT Co. Ltd. (Incheon, Korea). Oxidized MWCNT (ox-MWCNT) was obtained by dispersing 1 g of MWCNT in nitric acid (9 M) and stirring for 12 h, under reflux, at 150 °C. Then, they were filtered, washed repeatedly with ultrapure water until a neutral pH was obtained and dried for 24 h using a lyophilizer (Enterprise II, Terroni, Brazil) at −48 °C. Finally, the ox-MWCNT was autoclaved and dispersed in ultrapure water (0.5 mg mL^{-1}) by sonication for 60 min (Ultrasonic Bath, Cole-Parmer 08895-43, USA).

Thereafter, MWCNT and ox-MWCNT thermal stability was evaluated by thermogravimetric analysis (TGA) (STA, 449 F3 Jupiter@, NETZSCH, Germany) employing a heating rate of 5 °C min^{-1}, from room temperature to 750 °C, with a synthetic air flow of 50 mL min^{-1}. The structure and morphology of ox-MWCNT was observed by scanning electron microscopy (SEM) (MEV-EDS Inspect F50, FEI, UK) and transmission electron microscopy (TEM) (JEM-1400 plus, JEOL, USA) techniques. Chemical surface analyses were carried out on a K-Alpha photoelectron spectrometer (XPS) system (Thermo Fisher Scientific, UK) employing pass energies of 200 and 50 eV to obtain the survey and high-resolution spectra, respectively, and data were analyzed using Thermo Avantage software (Version 5.921) and the total oxidation degree was calculated as the ratio between the total content of oxygen-bonded carbon atoms (C—O, C═O and COO) and total sp^2 and sp^3 carbon atoms according to Padovani et al. (2015). The hydrodynamic sizes and zeta potentials were evaluated by dynamic light scattering (DLS) and electrophoretic light scattering (ELS) (Zetasizer Nano-Instrument, Malvern, UK), respectively.

2.2. Ox-MWCNT and cadmium co-exposure protocols

Two incubation protocols were conducted for ox-MWCNT interaction with $CdCl_2$ (purity 99.3%, J.T. Baker, Phillipsburg, USA): (A) Incubation Protocol A, the ox-MWCNT were incubated in RPMI/L-15 (Roswell Park Memorial Institute and Leibovitz's-15) medium + 10% FBS for 30 min (step 1) and, subsequently, the $CdCl_2$ were added and incubated for more 30 min (step 2); and (B) Incubation Protocol B, the ox-MWCNT were incubated with $CdCl_2$ for 30 min (step 1) and, thereafter, the RPMI/L-15 medium + 10% FBS were added and incubated for more 30 min (step 2). For all incubation protocols, thermoblock was used (Thermomixer C, Eppendorf, Germany) at 28 °C.

2.3. Determination of cadmium adsorption by MWCNT

Cadmium adsorption experiments were performed at 25 μg mL^{-1} of $CdCl_2$ and increasing concentrations of ox-MWCNT (0, 5, 10, 25, 50, 100 μg mL^{-1}) in order of each protocol A and B. After the incubation time the medium were centrifuged at 14000*g* and the supernatants were removed. Aliquots of 1 mL of supernatant were digested in triplicate in digester units using 4 mL of distilled HNO_3 at 100 °C for 12 h in a ceramic plate. Subsequently, the digested content was filtered at 45 μm, weighted and diluted up to twenty times with ultrapure water for analysis. Measurements of Cd content was performed using an inductively coupled plasma optical emission spectrometer (ICP-OES, iCAP 7000 Series, Thermo Scientific) with argon (99.9996%, White Martins-Praxair, SP, Brazil). Experiments were performed using HNO_3 (65%, Merck, Kenilworth, NJ, Brazil) and ultrapure water, resistivity higher than 18.5 MΩ cm, (Gehaka, Sao Paulo, Brazil).

Controls were prepared using the same protocol without sample (only reagents) in triplicate. Standard solutions used for ICP-OES calibration and for addition and recovery experiments were prepared by dilution of 1000 mg L^{-1} of Cd in 0.14 mol L^{-1} of HNO_3. The