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Evaluation of multiwalled carbon nanotubes toxicity in two fish species

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ABSTRACT

Carbon Nanotubes are among the most promising materials for the technology industry. Their unique physical and chemical proprieties may reduce the production costs and improve the efficiency of a large range of products. However, the same characteristics that have made nanomaterials interesting for industry may be responsible for inducing toxic effects on the aquatic organisms. Since the carbon nanotubes toxicity is still a controversial issue, we performed tests of acute and subchronic exposure to a commercial sample of multiwalled carbon nanotubes in two fish species, an exotic model (*Danio rerio*) and a native one (*Astyanax altiparanae*). Using the alkaline version of the comet assay on erythrocytes and the piscine micronucleous, also performed on erythrocytes, it was verified that the tested carbon nanotubes sample did not generate apparent genotoxicity by means of single/double DNA strand break or clastogenic/aneugenic effects over any of the species, independently of the exposure period. Although, our findings indicate the possibility of the occurrence of CNTs-DNA crosslinks. Apparently, the sample tested induces oxidative stress after subchronic exposure as shown by activity of superoxide dismutase and catalase. The data obtained by the activity levels of acetylcholinesterase suggests acute neurotoxicity in *Astyanax altiparanae* and subchronic neurotoxicity in *Danio rerio*.

1. Introduction

Carbon nanotubes (CNTs) are very interesting materials for technological innovation due to their remarkable physical and chemical properties. The deep understanding of the new phenomena emerging from these nanomaterials is leading to important breakthroughs in material, electronic, and biotechnology areas (Martinez et al., 2013).

The unique nanometer-scale structure of the CNTs is based on a graphene cylinder, typically a few nanometers in diameter, which can range in length from a few micrometers to millimeters. Single-walled carbon nanotubes (SWCNTs) consist of one such cylinder, and multi-walled carbon nanotubes (MWCNTs), as used in this study, comprises 2–50 such cylinders concentrically stacked with a common long axis. This structure gives the nanotubes an unusual combination of properties that are highly desirable in many industrial products (Poland et al., 2008).

However, the safety-related aspects of nanomaterials to the human health and the environment have not been well understood so far and the toxicity assessment of the CNTs is an important issue to be approached towards the development of a safe nanotechnology (Martinez et al., 2013).

The concern about the genotoxic effects of nanomaterials has grown in recent years. Genotoxicity induced by CNTs can be attributed to several factors, such as direct interaction of the particles with the DNA, indirect damage caused by reactive oxygen species (ROS), the release of toxic ions or direct interaction with cellular components (Wang et al., 2015, 2016; Girardi et al., 2016). Yet, only a few studies in the field of aquatic toxicology have approached the genotoxic effects of the CNTs in aquatic species. The genotoxic effects can affect the aquatic ecosystems for long periods, and the presence of genotoxic compounds in water can also impact non-aquatic species via food chains or simply as a result of drinking water (Filho et al., 2014).

Fish are often used as sentinel organisms for ecotoxicological studies because they play numerous roles in the trophic web, which they can rapidly absorb toxic substances and respond to low concentration of xenobiotics (Çavas and Ergene-Gözükara, 2005). In recent years, the

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zebrafish model (*Danio rerio*) has proven to be a simple model for the effective and rapid evaluation of nanoparticles toxicity (Duan et al., 2013; Filho et al., 2014). Likewise, *Astyanax* is a native genus of the southern Brazil basins that has been successfully used for ecotoxicology studies as demonstrated by Lemos et al. (2008), Silva et al. (2010), Erbe et al. (2011), Ramsdorf et al. (2012), and Freire et al. (2015).

The present study aimed to evaluate the acute and subchronic toxicity of a commercial sample of MWCNTs in an exotic fish model (*Danio rerio*) and in a native model (*Astyanax altiparanae*) using different biomarkers. To access genotoxicity effects, the alkaline version of the widespread comet assay was employed in combination with the miconucleous (MN) and nuclear abnormalities (NA) test. The hepatic toxicity was measured by the activity of Glutathione-S-Transferase (GST). Oxidative stress was measured by the activity of Superoxide Dismutase (SOD) and Catalase (CAT). To access neurotoxicity Activity of acetylcholinesterase (AchE) was applied.

2. Matherial and methods

2.1. Carbon nanotubes

A commercial sample of multiwalled carbon nanotubes (Sigma-Aldrich®, allotment 724769) was used to prepare a stock solution in distilled water at a concentration of 1 g/L. According to the manufacturer, the sample used was synthesized by chemical vapor deposition (with cobalt and molybdenum as catalysis) reaching 95% of purity. The informed average diameter, the diameter distribution and length are 6.6 nm, 6–9 nm and 5 μm, respectively.

For the characterization of the sample, X ray diffraction, Raman spectroscopy, thermogravimetric analysis and zeta potential were employed.

The X ray diffraction measures were obtained in a Shimadzu XRD 6000 diffractometer, using Cu-Kα (λ = 1,5418 Å) radiation, at 40 kV, 40 mA and 0,02° of resolution at 2 θ.

The Raman spectrum was obtained in a Renishaw Spectrophotometer coupled in an optical microscope at 1 μm of spatial resolution using 514 ηm laser with an excitation source of 20 mW. To achieve the spectrum, 30 scans were performed at 3500–200 cm^{-1} site at 10 s of running time.

The Zeta potential measures were obtained from a carbon nanotubes suspension in 2 mL of Milli-Q water from which it was withdrawn one aliquot of 500 μL and diluted in 15 mL of Milli-Q water. The resulting suspension was placed in ultrasonic bath (Ultraclear 1400 A, freq. US 40 kHz) for 15 min when, five measures were performed.

The thermogravimetric analysis was performed using 4 mg of the comercial sample in a Shimadzu DTG-60H. The sample was heated at heating rate of 10 °C/mim until 900 °C in N_2 atmosphere.

The metals quantification was reached employing the ICP-OES (Perkin Elmer Optima 8300) technique. The digestion protocol applied was the 3050B (U.S.EPA), using 0.21 g of multiwalled carbon nanotubes.

2.2. Experimental design

In order to evaluate the toxicity of the CNT, both species (*Danio rerio* and *Astyanax altiparanae*) were exposed to the samples through acute (96 h) and subchronic (21 days) contamination. The specimens of *D. rerio* and *A. altiparanae* were obtained in the specialized commerce. The fishes (at juvenile stage) were acclimatized for 30 days in tanks of 250 L (*A. altiparanae*) or 30 L aquaria (*Danio rerio*), with filtered water, constant aeration, average temperature of 26 °C, photoperiod of 12 h, and daily feeding.

To perform the acute exposure test, 60 specimens of *A. altiparanae* (1.5819 ± 0.7153 g) were distributed in four 30 L aquaria (15 each) filled with 21 L of filtrated water, among which, one of them was reserved for control, and the other 3 for the treatment. In addition, 40 specimens of *D. rerio* (0.3234 ± 0.082 g) were distributed in eight 2 L backers (with 5 each), then, two were reserved for control and the other 6 for the treatment (all duplicate). In both cases, the concentrations applied in the treatment were 0.5 mg/L; 5.0 mg/L and 50 mg/L. The contamination occurred through the water by the dilution of the stock solution without renovation of the contaminant along the experiment, and during all the 96 h the feeding was suspended.

To perform the subchronic exposure test, 60 specimens of *A. altiparanae* (1.9297 ± 0.8476 g) were distributed in four 30 L aquaria filled with 21 L of filtrated water (15 each), one of them was reserved for control and the other 3 for the treatment. Likewise, 48 specimens of *D. rerio* (0.3462 ± 0.0991 g) were distributed in eight 2 L backers (6 specimens each), so that, 2 were reserved for control and the others destined for the treatment (duplicate). The concentrations used in the treatments were 0.1 mg/L; 1.0 mg/L and 10 mg/L. The contamination occurred through the water and during all the 21 days, the contaminant was partially renewed (1/3) each 72 h. The feeding was kept always 24 h before each contaminant renovation.

Once the exposure period was concluded, the fishes were anesthetized in a solution with benzocaine 20%, measured, weighted and finally euthanized by making a section in the spinal cord. Blood was sampled trough heparinized capillaries (*A.* altiparanae) or through micropipette after decapitation (*D. rerio*). To measure the activity of biochemical biomarkers, samples of the livers and the brains were collected from the specimens of *A. altiparanae* and frozen at −80 °C. for further use as well as the bodies and heads of *D. rerio*.

The techniques used to manage the animals during the test and the procedures adopted to obtain samples of tissues, and organs were approved by the Ethical Commission of Animals Usage of the Universidade Tecnológica Federal do Paraná, under technical advice of number 2016/019.

2.3. Comet assay

The comet assay with peripheral blood (erythrocytes) was performed according to Speit and Hartmann (1999), modified by Cestari et al. (2004) and Ferraro et al. (2004). The blood was diluted in fetal bovine serum and kept under refrigeration for 24 h (protected from light) (Ramsdorf et al., 2009).

Microscope slides were prepared with a blood cell suspension (10 μL) in low melting point agarose (120 μL) at 37 °C, followed by incubation in lysis solution at 4 °C for 24 h. After lysis incubation, the slides were placed in a NaOH (10 M) and EDTA (200 mM) solution at a pH N 13 for 30 min for the DNA denaturation.

The electrophoresis was carried out at 25 V and 300 mA for 25 min at 4 °C, and slides were neutralized for 15 min with 0.4 M Tris, pH 7.5, fixed in 95% ethanol for 5 min, and stained with ethidium bromide (0.02 μg mL^{-1}).

For each fish, one hundred nucleoids were analyzed (Collins et al., 1997) according to the visual classification based on the migration of DNA fragments from the nucleus. The results were categorized into classes according to Collins et al. (1997): class 0 (no visible damage), class 1 (little damage), class 2 (medium damage), class 3 (extensive damage) and class 4 (maximum damaged). The score was calculated by multiplying the number of nuclei found in a class times the class number.

2.4. Piscine Micronucleus Test

The piscine micronucleus test (MNT) was performed according to the technique described by Hooftman and De Raat (1982). For each fish, 2.000 erythrocytes (for *A. Altiparanae*) or 1000 erythrocytes (for *D. rerio*) were examined under 1.000 × magnification and scored by the presence of both typical micronuclei and nuclear morphological abnormalities (NAs) manifested in changes in the normal elliptical shape of the nuclei. The frequency of micronuclei and nuclear morphological