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Histopathological alterations in the gills of Nile tilapia exposed to carbofuran and multiwalled carbon nanotubes

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ABSTRACT

Carbofuran is a nematicide insecticide with a broad spectrum of action. Carbofuran has noxious effects in several species and has been banned in the USA and Europe; however, it is still used in Brazil. Aquatic organisms are not only exposed to pesticides but also to manufactured nanoparticles, and the potential interaction of these compounds therefore requires investigation. The aim of this study was to examine the histopathological alterations in the gills of Nile tilapia (*Oreochromis niloticus*) to determine possible effects of exposure to carbofuran, nitric acid-treated multiwalled carbon nanotubes (HNO_3-MWCNTs) and the combination of carbofuran with nanotubes. Juvenile fish were exposed to different concentrations of carbofuran (0.1, 0.5, 2.0, 4.0 and 8.0 mg/L), different concentrations of HNO_3-MWCNTs (0.5, 1.0 and 2.0 mg/L) or different concentrations of carbofuran (0.1, 0.5, 2.0, 4.0 and 8.0 mg/L) with 1.0 mg/L of HNO_3-MWCNTs. After 24 h of exposure, the animals were removed from the aquarium, the spinal cord was transversely sectioned, and the second gill arch was removed for histological evaluation. Common histological changes included dislocation of the epithelial cells, hyperplasia of the epithelial cells along the secondary lamellae, aneurism, and dilation and disarrangement of the capillaries. All the groups exposed to carbofuran demonstrated a dose-dependent correlation in the Histological Alteration Index; the values found for carbofuran and carbon nanotubes were up to 25% greater than for carbofuran alone. This result indicates an interaction between these toxicants, with enhanced ecotoxic effects. This work contributes to the understanding of the environmental impacts of nanomaterials on aquatic organisms, which is necessary for the sustainable development of nanotechnologies.

1. Introduction

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranil methyl carbamate) is a nematicide insecticide with a broad spectrum of action and has been identified as a problematic chemical substance based on its use of chlordane, heptachlor and aldrin, according to the EPA (Environmental Protection Agency, 1976). Considering its noxious effects on birds, fish, mammals, insects, and aquatic invertebrates, carbofuran has been banned in the United States and Europe (USEPA, 2006); it is, however, still used in Brazil (Barbieri et al., 2013). The mode of action of carbofuran is related to the inhibition of acetylcholinesterase in the synaptic and neuromuscular junctions (Jash and Bhattacharaya, 1983).

In addition to being exposed to pesticides, aquatic organisms are also exposed to manufactured nanoparticles (Paschoalino et al., 2010; Handy et al., 2011; Martinez et al., 2013).

The industrial production and use of nanoparticles already amounts to several tons annually and will certainly increase. Nanomaterials are materials and products on a scale ranging from 1 to 100 nm (Masciangioli and Zhang, 2003; Roco, 2003; Moore, 2006; Smith, 2007) and have brought benefits to many sectors, including foodstuffs, electronics, pharmaceuticals, biotechnology, cosmetics and medical supplies, among others (Handy, 2008; Paschoalino, 2010). In addition, the surfaces of nanomaterials are of special interest in the development of new products (Handy et al., 2011), such as lighter and more resistant cages for aquaculture and more efficient filters for cleaning water, according to Handy et al. (2011).

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Increased production and use of large-scale nanoparticles increases concern about their toxicity because the risk of nanoparticles infiltrating different environmental elements (air, water and soil) is too high (Lovern et al., 2007).

The toxic effect of different nanomaterials in different organisms has been observed, but studies that measure the concentrations of nanomaterials in the environment are almost completely absent (Gottschalk et al., 2009).

The structural alterations observed in various tissues, structures and organs are general indicators of the health of fish and may be used to measure exposure to various anthropogenic pollutants (Hinton et al., 1992). The exposure of fish to chemical contaminants can induce numerous lesions in various organs (Sidermann, 1979; Bucke et al., 1996), and gills (Mallatti, 1985; Poleksic and Mitrovic-Tutundzic, 1994) are an appropriate organ for histological examination of the effects of pollutants in biological models.

The gills are responsible for vital functions such as gas exchange, ionic regulation, acid/base balance, the excretion of nitrogenated compounds and gustation (Machado and Fanta, 2003). The large surface area of the gills in direct and permanent contact with potential pollutants makes this organ a primary marker for aquatic pollution (Bernet et al., 1999). The gill is the first organ to react to unfavorable environmental conditions, and thus, morphological alterations of the gills represent responses to xenobiotics (Poleksic and Mitrovic-Tutundzic, 1994).

The skin and gills, organs in direct and permanent contact with pollutants, are the first barrier of the natural mechanisms of resistance to disease and pollution. These organs possess mucous cells, which play an important role in the resistance to diseases caused by pathogens and toxic substances, among other functions (Shephard, 1994). An increased number of mucus-secreting cells in the gill epithelium has been associated with the exposure of the organism to contaminants and is thus considered a histological alteration (Bernet et al., 1999). Another type of cell present in the gills is the chlorite cell. According to Evans et al. (2005), this cell type is the most active cell in fish gills, and it absorbs and secretes many ions and electrolytes and is greatly affected by various pollutants present in the environment, including pesticides (Khoshnood et al., 2010). Hypertrophy and hyperplasia, which correspond, respectively, to an increase in the volume of the cell and its proliferation (Meletti, 2003), are classified as two progressive alterations characterized by increased cell and tissue function and induced by alterations in cell physiology (Takashima and Hibiya, 1995).

The multifunctionality of gills, the vast surface area that they occupy and their location in intimate contact with the external environment makes the gills a key organ for the action of pollutants in the aquatic environment (Cettina, 2008). Thus, the histological alterations appear as a medium-term response to sublethal stressing agents, and histological techniques provide a rapid and precise method to detect the effects of pollutants, especially chronic exposure, in various tissues and organs (Johnson et al., 1993).

Studies have shown that pesticides are able to alter the gill tissue. Devi and Mishra (2013) observed gill alterations after exposure to organophosphate pesticide concentrations in *Channa punctatus*, and Ullah et al. (2015) observed these changes in *Tor putitoria* after exposure to cypermethrin. Exposure to nanomaterials also leads to brachial alterations. Federici et al. (2007) observed mainly edema and lamellar thickening after exposure to titanium dioxide nanoparticles (TiO2 NPs) in *Oncorhynchus mykiss*. Griffitt et al. (2007), analyzing the effects of the concentration of copper nanoparticles, observed proliferation of brachial cells and edema in *Danio rerio*. Nile tilapia (*Oreochromis niloticus*) is extensively cultured worldwide and is widely used in ecotoxicology studies to assess the effects of different contaminants on the gills (Rezende et al., 2014; Ayadi et al., 2015).

The present study was conducted to examine the effects of carbofuran, carbon nanotubes and the interaction between these two components on histological alterations of gill tissues in Nile tilapia (*O. niloticus*). The hypothesis of this study is that carbofuran and nitric acid-treated multiwalled carbon nanotubes (HNO_3-MWCNTs) interact to induce alterations in Nile tilapia gill tissues.

2. Materials and methods

Carbofuran (2,3-diidro-2,2-dimetil-7-benzofuranil metil carbamato) (99.5%, Sigma Aldrich) was used as the analytical standard. For the preparation of the stock solution, 100 mg of MWCNTs [Ctube 100, CNT Co. Ltd., Incheon - South Korea] were vacuum filtered through a 0.2 μm PTFE membrane after acid treatment under reflux with 9 mol/L HNO_3 (12 h at 150 °C). Nanotubes were then washed with deionized water until neutral pH of the filtrate was achieved and dried in vacuum for 24 h. This sample is called HNO_3-MWCNTs. This carbon nanotube sample was well characterized by Campos-Garcia et al. (2015) who described it as having the following characteristics: length < 5 μm [Field Emission Gun Scanning Electron Microscopy (FEG-SEM), FEI NanoLab], diameter distribution ranged from 10 to 40 nm [Transmission Electron Microscopy (TEM), Zeiss Libra 120], the surface area was 264 m^2/g [Brunauer–Emmett–Teller (BET) method, Micromeritics Instruments ASAP 2010], and surface charge was -27 mV (ζ-potential, nano-ZS Malvern instruments). Using Raman spectroscopy [TS-150 WITHEC spectrometer], we obtained the value of the I_D/I_G ratio (structural defect index) as 1.02, the decomposition temperature was 587 °C [Thermogravimetric analysis (TGA), TA Instruments SDT Q600], and the final content of metallic residues (iron oxide) in the HNO_3–MWCNT sample was less than 2.0% [Analytical microbalance, AD- 6 Perkin-Elmer].

The sample was prepared in a stock suspension of HNO_3–MWCNTs before being exposed to fish. For this experiment, 100 mg of HNO_3–MWCNTs was dispersed in 100 mL of deionized water and sonicated for 30 min [Ultrasound bath, Cole- Parmer 8891].

A total of 160 Nile tilapias with an average wet weight of 0.57 g ($\pm$ 0.10) and average total length of 2.36 cm ($\pm$ 0.30), produced at the Fisheries Institute in Cananéia on the southern coast of the State of São Paulo (Brazil), had their gills analyzed. Groups of 10 fish were put into 16 aquaria each with a capacity of 50 L and filled with 20 L of dechlorinated water with constant aeration. The pH and water temperature of all the aquaria were each measured for 24 h and were maintained at 7.25 ($\pm$ 0.11) and 19.9 °C ($\pm$ 0.3), respectively.

The groups were exposed, according to the LC50 values reported by Campos-Garcia et al. (2015), to the following carbofuran concentrations: 0.1, 0.5, 2.0, 4.0 and 8.0 mg/L; carbon nanotubes in the following concentrations: 0.5, 1.0 and 2.0 mg/L; and carbofuran in the concentrations: 0.1, 0.5, 2.0, 4.0 and 8.0 mg/L with 1.0 mg/L of HNO_3-MWCNTs added to them, as well as the respective controls. The concentrations selected were based on Campos-Garcia et al. (2015).

The *O. niloticus* were immediately removed from the aquarium after 24 h, weighed, and measured, and the spinal cord was transversely sectioned. The second gill arch was removed and fixed in iced McDowell (McDowell and Trump, 1976).

At the Institute of Biomedical Sciences of the University of Sao Paulo, the material was dehydrated and included in paraffin, and 4 μm thick slices were obtained using the microtome model Hyrax M25 of Zeiss®, for the preparation of slides. The slides were stained with Hematoxylin/Eosin (H./E.) and Periodic Acid-Schiff (P.A.S.)