Comparative Biochemistry and Physiology, Part C 218 (2019) 30–35

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

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

Neurotoxicity in zebrafish exposed to carbon nanotubes: Effects on neurotransmitters levels and antioxidant system☆

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ARTICLE INFO

Keywords:
Nanotoxicology
Nanomaterials
Fish
Neurotransmitters
Neurotoxicity

ABSTRACT

Given the increasing use of carbon nanotubes (CNT) in several industries and technological applications, it is essential to perform in vivo toxicological studies with these nanomaterials to evaluate their potential ecotoxicity. Dopamine (DA) and serotonin (5HT) are key neurotransmitters for brain functions and behavioral responses. Determination of DA and 5HT were performed in brain samples from zebrafish *Danio rerio* exposed i.p. to single-walled CNT (SWCNT), besides analyzing acetylcholinesterase (AChE) and ectonucleotidases activity, lipid peroxidation and total antioxidant capacity. Results showed that treatment with SWCNT increased between 3 and 6-fold the concentration of DA and 5HT ($p < 0.05$). Similarly, a significant reduction ($p < 0.05$) in AChE activity was observed in the brains of SWCNT exposed zebrafish when compared to the control groups. Cholinergic, serotonergic, and dopaminergic systems, through AChE activity and serotonin and dopamine levels, respectively were affected by SWCNT in the zebrafish brain. Alterations in these neurotransmitters can potentially affect several physiological and behavioral that they control.

1. Introduction

Carbon nanotubes (CNT) have unique physical, chemical, electrical, and mechanical properties that offer many potential applications and, because of these, a large-scale production of CNT is increasing. In this context, it is expected that fauna, flora, and human will be exposed to CNT (Liu et al., 2012).

Nowadays, nanotechnologies and the applications of nanomaterials have an unquestionable importance, being responsible to increase the consumables, products of medical devices, biosensors, and drug delivery (Kunzmann et al., 2011). However, it is essential to conduct in vivo assays to know whether these nanomaterials are responsible or not for cell/tissues perturbations and diseases. On the order hand, it is difficult to analyze inherent CNT toxicity because of their chemical and structural complexity, including surface charge, shape, length, agglomeration, and layer number (Liu et al., 2012).

Only small lipophilic molecules of < 500 Da can across the blood-brain barrier (BBB), including amino acids, hexoses, neuropeptides, and proteins, which are transported into the brain via specific carriers (Wohlfart et al., 2012). Regarding carbon nanotubes, it is still not known exactly whether they can cross the BBB, although there are studies that suggest this possibility as noted by Yang et al. (2007). The authors showed that single-walled carbon nanotubes (SWCNT) were distributed in the entire body of mice, including the brain, after 28 days of exposure, indicating that SWCNT could overcome the BBB enter, being able to reach the brain and cause damage.

☆ This article is part of a special issue entitled: Aquaculture- edited by Dr. John Stieglitz and Dr. Martin Grosell.

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https://doi.org/10.1016/j.cbpc.2018.12.008
Received 28 September 2018; Received in revised form 3 December 2018; Accepted 6 December 2018
Available online 11 December 2018
1532-0456/

In fish, no studies have been published, to the best of our knowledge, reporting the presence of carbon nanotubes in the brain. For example, Weber et al. (2014) did not find pegylated SWCNT in the brain of i.p. exposed zebrafish, but they reported brain histopathologies, suggesting that this CNT possess the potential to induce neurotoxicity through direct or indirect pathways. A recent study by Seixas et al. (2018) observed induction of protein carbonyl groups in brains of zebrafish exposed to SWCNT and multi-walled carbon nanotubes (MWCNT) through diet. To sum up, it is not clear in fish species if CNT damage is caused directly by carbon nanotubes that cross the BBB or if any changes occur indirectly such as physical interference of SWCNT with cellular and extracellular constituents which may cause alterations of vital cellular processes, leading to various degrees of cellular injury and, in some cases, even to cell death (Shvedova et al., 2012).

The cholinergic system also appears to be altered in presence of SWCNT and MWCNT, being reported acetylcholinesterase (AChE) inhibition (Wang et al., 2009a, 2009b). This enzyme hydrolyzes the neurotransmitter acetylcholine (ACh), one of the most crucial enzymes for nerve response and function. Its inhibition causes the accumulation of ACh, interfering in the control of many physiological and behavioral responses in animals (Worek et al., 2002).

The purinergic signaling system includes ATP, a triphosphate nucleotide that exists in all cells and is involved in the regulation of many physiological processes in the extracellular medium where this extracellular ATP can be hydrolyzed through a cascade of a variety of enzymes called ectonucleotidases, which are anchored in the cell membrane, converting ATP to adenosine (Burnstock, 2012; Senger et al., 2005). The hydrolysis of ATP to AMP is catalyzed mainly by an ectonucleotidase named nucleoside triphosphate diphosphohydrolase (NTPDases) and nucleotide AMP is hydrolyzed to adenosine by the action of an ecto-5′-nucleotidase (Zimmermann, 2011).

In this study, it was evaluated zebrafish dopaminergic, serotonergic, cholinergic, and purinergic systems treated with SWCNT by analyzing DA and serotonin (5HT) levels and acetylcholinesterase ectonucleotidases activity. It was also analyzed antioxidant capacity and lipid peroxidation in brain of zebrafish treated with SWCNT, once significant evidence indicates that oxidative stress plays a central role in CNT toxicity, including fish (Shvedova et al., 2012; da Rocha et al., 2013; Seixas et al., 2018). However, there is a lack of information in terms of neurotoxic effects that CNT and other carbon nanomaterials can exert on aquatic organisms, data that should be valuable considering the potential consequences not only at the biochemical/physiological level but also in terms of behavioral impact.

2. Materials and methods

2.1. Fish care

All procedures are in accordance and were approved by the Ethics Committee on Animal Use (CEUA) of the Universidade Federal of Rio Grande – FURG (process number 23116.002327/2013-47). Adult zebrafish *Danio rerio* (mean weight: 0.52 ± 0.1 g) were purchased from a commercial supplier (Red Fish, RS, Brazil) and were acclimated at least for two weeks during which they were fed for two times a day with a commercial diet (NOVOBEL, JBL). No more than 60 fish were placed in each of the 60 l tanks. The pH and water temperature were fixed in 7.5–8.0 and 28 °C, respectively. Tank maintenance included a 1/3 water change three times a week after removal of the excess of food or fish waste from the bottom using a siphon.

2.2. Synthesis and characterization of SWCNT

The employed SWCNT were of the same batch employed in the study of da Rocha et al. (2013) and details of the synthesis method employed as well as the characterization employing Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), and Thermogravimetric Analysis (TGA) can be found in that article. The first step of purification of produced SWCNT was performed using concentrated HCl that rendered a product with around 10% in weight of impurities. In the second step of purification it was used a concentrated mixture of HNO_3 and H_2SO_4 (3:1) that generated SWCNT with 0.3% of impurities in weight (mainly nickel, iron, and yttrium oxides). The second step of purification used a concentrated mixture of HNO_3 and H_2SO_4 (3:1) that rendered SWCNT with 0.3% of impurities in weight.

An additional morphological characterization was performed through Raman spectroscopy, using a Renishaw Model inVia Spectrometer. The experiments were performed at room temperature, in the range 0–3100 cm^{-1} using a laser of 532 nm wavelength.

2.3. Preparation of SWCNT suspensions

SWCNT suspensions were prepared through sonication (sonication power: 9.3 W, energy input: 16.7 kJ at 25 °C using a Ultronique/Eco-sonics Q-3.0/40A sonicator) with the detergent sodium dodecyl sulphate (SDS) (3 g/L) and Milli-Q water during 3 h (Smith et al., 2007; da Rocha et al., 2013).

2.4. SWCNT exposure

Adult zebrafish were acclimated under the same conditions described in Section 2.1. Fish were anesthetized through immersion in benzocaine (1 mM) and then 10 μL of SWCNT suspension (30 mg/kg) was intraperitoneally (i.p.) injected, following a previous study of da Rocha et al. (2013). In control groups, fish were injected with 10 μl of SDS detergent used to prepare SWCNT suspension. To observe if the benzocaine anesthetic could promote some unwanted effect on the brain, some anesthetized zebrafish were selected as a second control group (benzocaine control), as performed in a previous study (da Rocha et al., 2013). A third control group was constituted by fish that were injected with Milli Q, the same employed to prepare the SWCNT suspension.

Immediately after the anesthesia and/or injection procedure, zebrafish were placed in constant aeration until they regain normal activity of swimming. All treated fish were maintained in glass aquariums in an incubator under the same conditions of the acclimation period (Section 2.1). After 24 h, the treatments were repeated, and after 48 h all the fish were euthanized and their brains dissected for analysis. Fish were not fed 24 h prior to or during the experiment. After dissection, pools of five fish brains were used to compose one sample for each measurement and then the brain samples were stored at −80 °C. All measurements were performed in triplicate, employing four pools ($n = 4$) of five zebrafish brains in each treatment.

2.5. Total antioxidant capacity

Brains were homogenized (1:5 w/v) in a Tris–HCl buffer (100 mM, pH 7.75) with EDTA (2 mM) and Mg2+ (5 mM) (da Rocha et al., 2009). The homogenates were centrifuged at 10,000 × *g* for 20 min at 4 °C) and the supernatants resulting from this centrifugation were analyzed. Total protein content was performed with a commercial kit based on the Biuret method, using a microplate reader (Biotek ELX 800) at a wavelength of 550 nm. Triplicate measurements were performed, presenting a variation coefficient of 5% or lower. Total antioxidant competence against peroxyl radicals was evaluated through ROS determination in brain samples of fish treated or not with a peroxyl radical generator, 2,2′-azobis 2 methylpropionamidine dihydrochloride (ABAP; 4 mM; Aldrich), according to the methodology proposed by Amado et al. (2009). Further details can be found in da Rocha et al. (2009). The relative difference between ROS area with and without ABAP is an estimate of total antioxidant capacity against peroxyl radicals, with high area difference meaning low antioxidant capacity and vice versa:

low area difference implies in high antioxidant capacity against peroxyl radicals in samples (Amado et al., 2009).

2.6. Measurement of lipid peroxidation

Brains were homogenized (1:10) in KCl 1.15% plus 35 mM of butylated hydroxytoluene (BHT). Lipid peroxidation was measured through determination of thiobarbituric acid reactive substances (TBARS), following the methodology of Oakes and Van der Kraak (2003) and adapted to a microplate reader by da Rocha et al. (2009). The fluorescence was registered after excitation at 520 nm and emission of 580 nm. The concentration of TBARS (nanomoles/mg of wet tissue) was calculated employing tetramethoxypropane (TMP) as a standard.

2.7. Determination of dopamine and serotonin levels

The levels of 5HT and DA in samples were analyzed by a high-performance liquid chromatography system (ESA Coulochem III, Bedford, MA) composed by two pumps (ESA Model 584 HPLC Pump, Shimadzu, Kyoto, Japan), a communication bus module (ESA Model 582 LPG, Shimadzu) and coulometric electrochemical detector. The detection was performed using a conditioning cell (ESA model 5020) that was maintained at a potential of +900 mV and a dual analytical cell (ESA model 5011A) fixed at 50 mV (first cell) and 700 mV (second cell). Brains from zebrafish i.p., injected with SWCNT were homogenized (1:5 volumes) in the mobile phase (95% potassium phosphate dihydrogen 25 mM, 0.1% formic acid, pH 2.9 and 5% acetonitrile), and centrifuged (5000 ×*g*) for 15 min, at 4 °C. The supernatant fraction was filtered and injected (20 µl) directly to the HPLC system. The analytes were separated on an ACE LC18 column (250 × 4.6 mm, 5 µm) at an isocratic flow of 0.9 mL/min, and the peak identification and quantification of 5HT and DA were performed using the software EasyChrom (Agilent Technologies, Santa Clara, CA). Under specified conditions, dopamine clearly eluted in about 5 min, whereas serotonin took ~10 min to elute. Levels of DA and 5HT in the samples were calculated based on standard calibration curves of dopamine and serotonin (analytical standards from Sigma, purity ≥ 98%), and the concentration of monoamines was expressed as picograms per milligram of tissue.

2.8. Measurement of acetylcholinesterase activity

Zebrafish brains were homogenized on ice in 60 volumes (v/w) of 50 mM Tris– HCl, pH 8.0, in a glass-Teflon homogenizer. Acetylcholinesterase activity was measured as the method described previously (Ellman et al., 1961) determining the rate of hydrolysis of acetylthiocholine (ACSCh, 0.8 mM) in 2 ml assay solutions with 100 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB. Samples containing protein (10 µg) and the reaction medium described above were pre-incubated for 10 min at 25 °C followed by starting of reaction with the addition of substrate. Substrate hydrolysis was monitored through the generation of the thiolatedianion of DTNB at 412 nm every 30 s for 2–3 min. The linearity of absorbance towards time and protein concentration was previously determined. Acetylcholinesterase activity was expressed as micromoles of thiocholine (SCh) released per hour per milligram of protein.

2.9. Ectonucleotidase assays

NTPDases and ecto-5′-nucleotidase assays were performed as described previously (Senger et al., 2005). Zebrafish brain membranes (3 µg protein for NTPDase and 5 µg protein for ecto-5′-nucleotidase) were added to the reaction medium containing 50 mM Tris-HCl (pH 8.0) and 5 mM CaCl2 (for the NTPDase activity) or 50 mM Tris-HCl (pH 7.2) and 5 mM MgCl2 (for the ecto-5′-nucleotidase activity) at a total volume of 200 µl. The samples were pre-incubated for 10 min at 37 °C and the reaction was initiated by the addition of substrate (ATP, ADP or AMP) to a final concentration of 1 mM. After 30 min, the reaction was stopped by the addition of 200 µl of 5% trichloroacetic acid. The samples were kept on ice for 10 min. Samples received 1 ml of a colorimetric reagent composed of 2.3% polyvinyl alcohol, 5.7% ammonium molybdate, and 0.08% malachite green. After 20 min, the quantification of inorganic phosphate (Pi) released was determined spectrophotometrically at 630 nm and the specific activity was expressed as nmol of Pi released per min per mg of protein. To correct non-enzymatic hydrolysis of the substrates, it was employed controls putting the enzyme preparation after the addition of trichloroacetic acid. Incubation times and protein concentrations were chosen to ensure the linearity of the reactions.

2.10. Statistical analysis

Values of all measurements were expressed as mean ± 1 standard error. Statistical analysis was performed through the analysis of variance (ANOVA) followed by Newman–Keuls test or orthogonal comparisons. Previously, the assumptions of normality and homogeneity of variance were verified and logarithmic transformation was applied if at least one of assumptions was violated (Zar, 1984). In all cases, the significance level was fixed in 5%.

3. Results

The presence of the characteristic bands of CNT is evidenced in Raman spectra shown in Fig. 1. In the spectral region, three bands are observed. These bands indicate the band of graphite (G band - about 1600 cm^{-1}) and the band of disorder and defects in the structure (D band - about 1380 cm^{-1}). The peak related to the structure of graphite (G') at about 2700 cm^{-1} is also evident. The previous study of da Rocha et al. (2013) that employed the same batch os SWCNT showed by SEM that the HCl and HNO_3 + H_2SO_4 purification steps were efficient for the removals of by-products that were detected by TGA.

Lipid peroxides levels (Fig. 2a) showed no statistically significant difference ($p > 0.05$) among the experimental groups. However, total antioxidant capacity against peroxyl radicals was statistically significant higher (lower relative area) ($p < 0.05$) in brains from zebrafish treated with SWCNT when compared with brains coming from zebrafish of the different control groups (Fig. 2b).

Results of Fig. 3. show the significantly increased levels of 5HT and DA in brains of zebrafish treated with 30 mg of SWCNT/kg compared

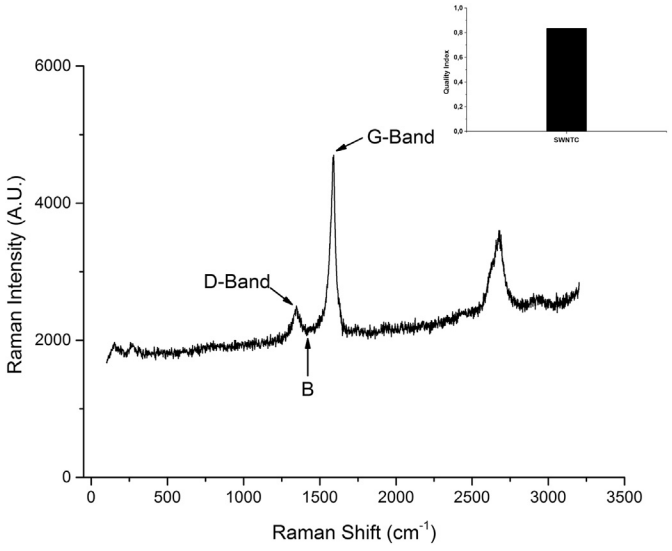

Fig. 1. Raman spectra of SWCNT showing a distinct peak at about 1.600 cm^{-1} (G-band), corresponding to the graphitic stretch of SWCNT.

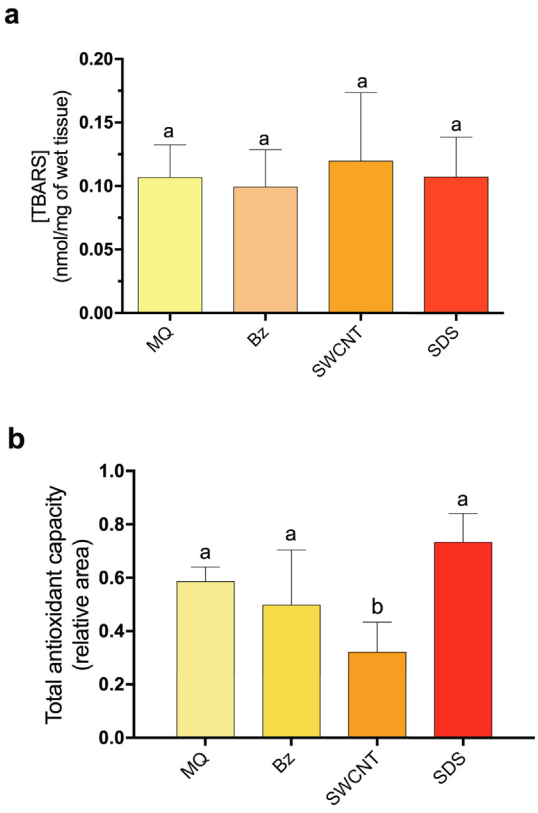

Fig. 2. The concentration of thiobarbituric acid reactive substances (TBARS) (a) and total antioxidant capacity against peroxyl radicals (ACAP) (b) in brains from zebrafish exposed during 48 h to different treatments. Milli Q: fish that were anesthetized and injected with Milli Q water. Bz: fish that were anesthetized but not injected. SWCNT: fish that were anesthetized and injected with single-walled nanotubes suspension. SDS: fish that were anesthetized and injected with the detergent. Data are expressed as mean + 1 standard error ($n = 4$) Different letters indicate statistical differences ($p < 0.05$). To ACAP a relative area was calculated by dividing area difference (with and without ABAP) by area without ABAP (background area). Larger area means lower antioxidant capacity.

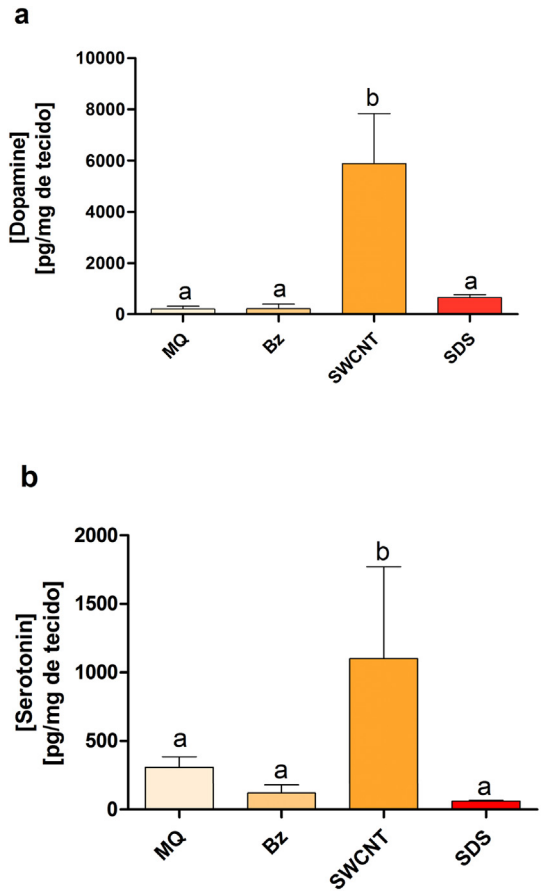

Fig. 3. Dopamine (DA) (a) and serotonin (5HT) concentration (b) in brains from zebrafish exposed during 48 h to different treatments. Milli Q: fish that were anesthetized and injected with Milli Q water. Bz: fish that were anesthetized but not injected. SWCNT: fish that were anesthetized and injected with single-walled nanotubes suspension. SDS: fish that were anesthetized and injected with the detergent. Data are expressed as mean + 1 standard error ($n = 4$) Different letters indicate statistical differences ($p < 0.05$).

with control groups (benzocaine and SDS treatments) after 48 h exposure ($p < 0.05$). Both 5HT and DA basal levels were around 3 to 6-fold greater in brain treated with SWCNT than in controls (Fig. 3a and b, respectively).

Acetylcholinesterase (AChE) activity decreased in brains from zebrafish treated with SWCNT when compared with the control group ($p < 0.05$) (Fig. 4).

Brains from zebrafish treated with SWCNT did not present altered levels of ATP ($p > 0.05$) (Fig. 5a) as well as in the other two nucleosides AMP and ADP (Fig. 5b and c, respectively) ($p > 0.05$).

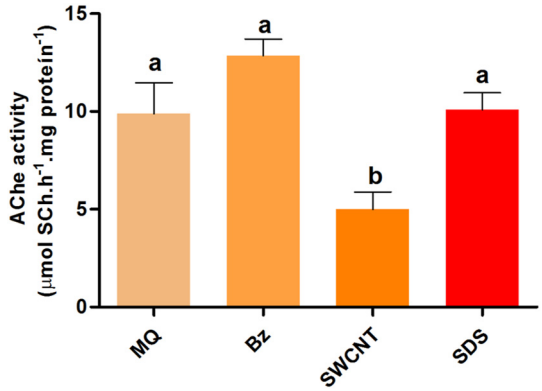

Fig. 4. Acetylcholinesterase (AChE) activity in brains from zebrafish exposed during 48 h to different treatments. Milli Q: fish that were anesthetized and injected with Milli Q water. Bz: fish that were anesthetized but not injected. SWCNT: fish that were anesthetized and injected with single-walled nanotubes suspension. SDS: fish that were anesthetized and injected with the detergent. Data are expressed as mean + 1 standard error ($n = 4$). Different letters indicate statistical differences ($p < 0.05$).

4. Discussion

The intrinsic toxicity of carbon nanotubes has been attributed to their physic-chemical characteristics as their smallness and the remarkably large surface area per unit mass and high surface reactivity (Zhao and Liu, 2012). This reactivity is correlated with the ability of nanomaterials like SWCNT to trigger the generation of ROS that can promote oxidative stress, favoring the generation of lipid peroxidation (Shvedova et al., 2012). However, in this study, we did not observe higher lipid peroxidation in brains from zebrafish treated with SWCNT and the control. On the other hand, total antioxidant capacity against peroxy-radicals was statistically significantly higher in the brains from zebrafish treated with SWCNT than in controls. Frequently, to preserve the overall homeostatic redox balance in presence of some xenobiotic substances, cells employ primary strategies as the induction of the antioxidant system (Monserrat et al., 2007). In this way, it is possible that the antioxidant response should cope with a pro-oxidant condition,

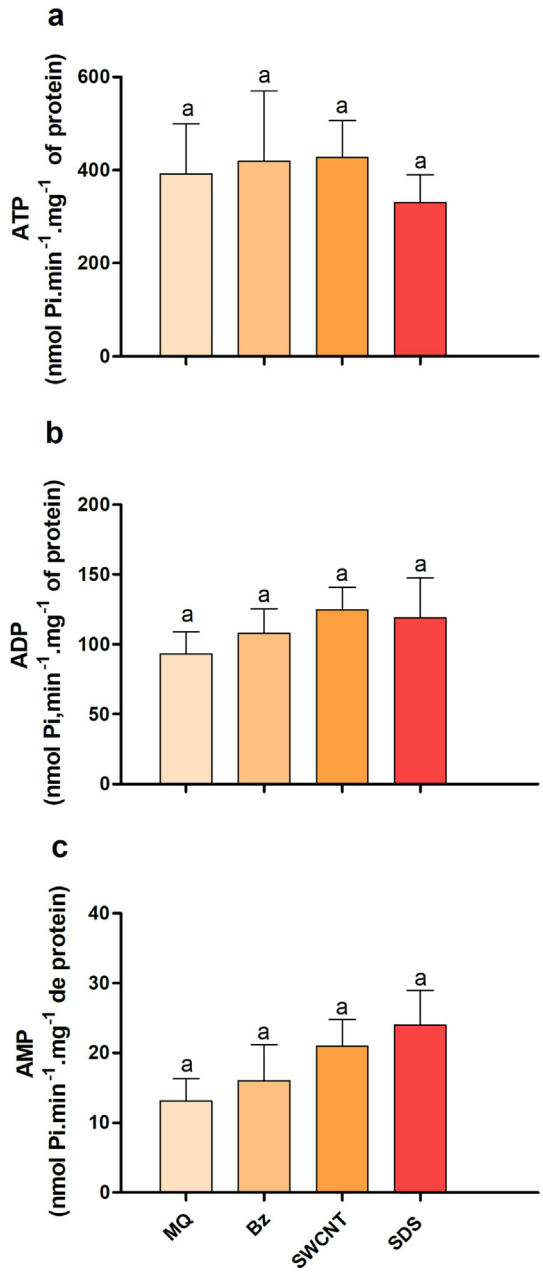

Fig. 5. Effect of different treatments on ATP (a), ADP (b) and AMP (c) hydrolysis in brains from zebrafish exposed during 48 h to different treatments. Milli Q: fish that were anesthetized and injected with Milli Q water. Bz: fish that were anesthetized but not injected. SWCNT: fish that were anesthetized and injected with single-walled nanotubes suspension. SDS: fish that were anesthetized and injected with the detergent. Data are expressed as mean + 1 standard error ($n = 4$) Different letters indicate statistical differences ($p < 0.05$).

adding to maintain almost constant lipid peroxides levels. This result also fits with a previous study from our research group, where no lipid damage in the brains from zebrafish treated with SWCNT was also observed (da Rocha et al., 2013).

Neurotransmitters such as dopamine (DA), serotonin (5HT) and acetylcholine (ACh) are essential regulators of brain functions (Nilsson, 1990; Almeida et al., 2003) and alterations of neurotransmitters and their metabolites has been used as an indicator of toxicity in the central nervous system (CNS) (Wang et al., 2009a, 2009b; Powers et al., 2011). As in the case of increased DA and 5HT levels found here, intranasal instilled copper nanoparticles (Cu NPs) in mice increased DA levels in the striatum, cerebral cortex, cerebellum, and the hippocampus. In addition, the intranasal instilled Cu NPs stimulated 5HT secretion in the hippocampus, cerebral cortex, and striatum (Zhang et al., 2012). In another study with mice, significant increases in the amount of DA and its metabolites were observed in the striatum and prefrontal area of the nanoTiO2 exposed group compared to the control animals (Takahashi et al., 2010). Some studies have observed variations in brain 5HT as Shams et al. (2015) that found that social isolation reduced anxiety and 5HT levels in this species. Alcohol also has been reported to reduce 5HT brain content in zebrafish showing low activity levels (Tran et al., 2016).

Studies have suggested that accumulation of dopamine in the cytosol can lead to oxidative stress and neurotoxicity (Goldstein et al., 2012; Masoud et al., 2015). Although we observed in this study a significant increase in the dopamine and serotonin neurotransmitters, we noticed no significant increase in the levels of lipid peroxidation. One might speculate that the absence of oxidative stress parameters response occurred due to the low sensibility of the fluorometric assay employed to measure MDA (product from the oxidative degradation of polyunsaturated fatty acids) employed in this work. In a study comparing MDA detected by HPLC (High-performance liquid chromatography) and by fluorimetry, it was measured TBARS in serum from 250 men and women healthy and the authors found a total mean value of 1.086 ± 0.43 μmol/l using the HPLC method and 0.135 ± 0.04 μmol/l using fluorometry (Seljeskog et al., 2006). Therefore, since previous studies have suggested that cytosolic dopamine is highly reactive and can induce lipid peroxidation (Goldstein et al., 2012; Kim and Kang, 2015; Masoud et al., 2015), it would be interesting testing the brain samples treated with SWCNT by HPLC.

Acetycholinesterase (AChE) controls a large proportion of physiological and behavioral responses, so any changes to these regulatory abilities could be potentially harmful to fish and a considerable reduction in acetylcholinesterase activity is frequently observed in fish exposed to toxic substances, pesticides, insecticides, metals, organochlorines, and herbicides (Jebali et al., 2006; Monserrat et al., 2007; Modesto and Marinez 2010; Kim and Kang, 2015). SWCNT and MWCNT had a high affinity for AChE, causing 76–88% inhibition of AChE as observed in an in vitro study (Wang et al., 2009a, 2009b). As consequence of AChE inhibition, ACh accumulates, promoting an abnormal content of this neurotransmitter, and interfering with the function of the nervous system and eventually leading to respiratory failure and death (Worek et al., 2002). Inhibition of this enzyme has been considered a toxic specific biomarker for organophosphorus pesticides although, as discussed by Jebali et al. (2006), several other molecules can inhibit this enzyme.

In a research using Xenopus larvae, the organisms were exposed for 12 days to DWCNT (double-walled carbon nanotubes) presented black masses in gills whatever the concentration (10 and 50 mg/l of raw DWCNT), thus promoting branchial obstruction, potentially generating gaseous exchanges perturbations and/or anoxia (Mouchet et al., 2010). In our study, even though we did not observe a significant rise in lipid damage, an increase of antioxidant capacity was registered. These findings were surprising because antioxidant improvement is a physiological response to an ongoing oxidative stress (Hermes-Lima et al., 2015).

ATP is an important signaling molecule, which can play an unmatched role in synaptic transmission, acting as a neurotransmitter (Burnstock, 2012) co-released with other signaling molecules, like glutamate, GABA, and ACh in different sub-populations of neurons in the central nervous system (Nakanishi and Takeda, 1973). Results indicated that ATP, ADP and AMP hydrolysis in zebrafish treated with SWCNT were not altered in any concentration tested on brain membranes. As far as we know, the possible toxic effects caused by nanotubes exposure on the enzymes involved in the purinergic system have not yet been investigated in any species. For that reason, it is vital more studies, including different time, exposure pathways, and a large range of carbon nanotubes concentrations, to achieve a better compression

about SWCNT toxicity mechanisms in the central nervous system.

Main conclusions of the present study indicate that SWCNT does not regulate the purinergic system. In the other hand, cholinergic, serotonergic, and dopaminergic systems, through AChE activity and serotonin and dopamine levels, respectively, were affected by SWCNT in zebrafish brain, results that can affect several physiological and behavioral responses in this organism.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

Da Rocha, AM received a fellow from Brazilian agency CAPES and we would like to thank the laboratory technicians working at the Instituto de Ciências Biológicas (ICB). We are grateful to Instituto Nacional de Ciência e Tecnologia Nanomaterial de Carbono and to Rede de Nanotoxicologia (MCTI/CNPq) (Proc. 421701/2017-0) for financial and logistic support. Carla D. Bonan, Daniela Martí Barros, Eduardo Alves de Almeida, José Marìa Monserrat, and Mauricio Reis Bogo are productivity research fellows from the Brazilian National Council of Scientific and Technological Development (CNPq).

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