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FISIOLOGIA ANIMAL COMPARADA

EMBRIO E NEUROTOXICIDADE DE NANOTUBOS DE CARBONO E FULEROL EM ZEBRAFISH *Danio rerio* (Teleostei, Cypridinae)

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Tese defendida no âmbito do Programa de Pós-Graduação em Ciências Fisiológicas – Fisiologia Animal Comparada, como parte dos requisitos para obtenção do título de doutor em Ciências Fisiológicas.

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“A perda das ilusões a respeito de uma situação é a primeira condição para se sair de uma situação da qual se necessite de ilusões”.

Karl Marx

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Lista de Abreviaturas

ABAP: 2,2’ azobis 2 metilpropionamidina dihidrocloreto

ACAP: capacidade antioxidante contra peróxi-radicais

ACh: acetilcolina

AChE: aceticolinesterase

ADP: adenosina difosfato

AgNPs: nanopartículas de prata

AHR: translocador nuclear receptor aril hidrocarboneto

AMP: adenosina monofosfato

ARE: elementos de resposta antioxidante

ATP: adenosina trifosfato

ATF4: fator de ativação de transcrição 4

atp5f1: subunidade b da ATP sintase mitocondrial

BHT: hidroxitolueno butilado

CAT: catalase

cDNA: DNA complementar

Cu-NPs: nanopartículas de cobre

CYP1: citocromo P450 1

$C_{60}(OH)_n$: fulerol

DNA: ácido desoxirribonucleico

DOPA: dopamina

DWCNT: nanotubos de carbono de parede dupla

eif1b: fator de iniciação de tradução eucariótica

ERO: espécies reativas de oxigênio

GA: goma arábica

GABA: ácido γ-aminobutírico

GCL: γ- glutamilcisteína ligase/glutamato cisteína ligase

GCLc: subunidade catalítica da glutamato cisteína ligase

GCLr: subunidade regulatória da glutamato cisteína ligase

GPx: glutationa peroxidase

GPx1: glutationa peroxidase 1

GSH: glutationa reduzida

GST: glutationa S-transferase

GST p2: glutationa S-transferase pi 2

hpf: horas pós fertilização

H_2O_2: peróxido de hidrogênio

i.p.: injeção intraperitoneal

Maf: proteína de fibrossarcoma músculo-aponeurótico

MPP: 1-metil-4-fenilpiridínio

MWCNT: nanotubo de carbono de parede múltipla

NF-B: fator nuclear B

NM: nanomateriais

npTiO_2: nanopartículas de titânio

Nrf2: fator nuclear eritróide 2 relacionado ao fator 2

NTC: nanotubos de carbono

NTPDases: nucleosídeo trifosfato difosfohidrolase

PARP1: poli-ADP ribose polimerase-1

PAHs: hidrocarbonetos aromáticos policíclicos

RT-PCR: reação em cadeia da polimerase via transcripatese reversa

SEM: microscopia eletrônica de varredura

SDS: dodecil sulfato de sódio

SiO_2-NPs: nanopartículas de sílica

SOD: superóxido dismutase

SWCNT: nanotubo de carbono de parede simples

TEM: microscopia eletrônica de transmissão

TBARS: determinação de substâncias reativas ao ácido tiobarbitúrico

TiO_2-NPs: nanopartículas de dióxido de titânio

ywhaqb: proteína de ativação da tirosina 3-monooxigenase triptofano

5HT: serotonina

6-OHDA: 6-hidroxidopamina

1. Resumo

Em contrapartida à produção crescente do uso nanomateriais na indústria, ainda são poucos os estudos acerca da toxicidade dos nanomateriais. Os nanotubos de carbono podem prejudicar a estrutura das membranas biológicas e serem possíveis fontes de toxicidade. Por outro lado, nanomateriais que passam por modificações químicas (funcionalização) podem aumentar sua solubilidade, como é o caso do fulerol, um derivado do fulereno que em alguns modelos animais, possui efeito neuroprotetivo, antiapoptótico e antimicrobiano. Diante disso, estudamos os efeitos de nanotubos de carbono de paredes simples (SWCNT) e fulerol administrados via injeção intraperitoneal (i.p.) em adultos de zebrafish *Danio rerio* (Teleostei, Cypridinae) a fim de analisar possíveis alterações nos parâmetros de estresse oxidativo no cérebro destes organismos. Além disso, foram avaliadas prováveis interferências em neurotransmissores importantes dos sistemas dopaminérgico, serotonérgico e colinérgico, após tratamento via IP de zebrafish adultos com SWCNT e na atividade de ectonucleotidases, bem como a expressão de alguns genes relacionados a resposta antioxidante, tanto em *Danio rerio* adulto quanto em embriões de mesma espécie expostos a SWCNT. Após análises, foram observadas mudanças evidentes na expressão de genes como o fator de transcrição Nrf2 e as subunidades catalítica e regulatória da enzima glutamato cisteína ligase em cérebros de zebrafish tratados com SWCNT ou fulerol via i.p. Genes considerados normalizadores foram modificados quando embriões foram tratados com SWCNT. Além disso, alterações na capacidade antioxidante total e concentrações aumentadas de neurotransmissores como dopamina e serotonina foram observadas em cérebro de zebrafish tratados da mesma maneira com SWCNT. Frente

a esses resultados, podemos concluir que nanomateriais de carbono exercem influência sob o sistema neurológico de zebrafish através de mudanças evidentes em neurotransmissores responsáveis por depressão e ansiedade, bem como em nível fisiológico, por meio da regulação na expressão de importantes genes na defesa antioxidante.

PALAVRAS-CHAVE: nanomateriais, nanotoxicologia, estresse oxidativo, sistema antioxidante, zebrafish *Danio rerio*, neurotransmissores.

2. Abstract

In contrast to the high production of nanomaterials used in the industry, there are few studies on the toxicity of nanomaterials. Among them may be mentioned carbon nanotubes, which may damage to the structure of biological membranes and to be potential sources of toxicity. Moreover, nanomaterials that are related to chemical changes (functionalization) can increase its solubility, such as the fulerol, a derivative of fullerene, which in some animal models have neuroprotective, anti-apoptotic and anti-microbial effect. Therefore, we studied the effects of single walled carbon nanotubes (SWCNT) and fulerol administered via intraperitoneal injection (i.p.) in adult zebrafish *Danio rerio* (Teleostei, Cypridinae) to examine possible changes in parameters of oxidative stress in the brains of organisms. Furthermore, possible interference were evaluated in the dopaminergic, cholinergic and serotonergic neurotransmitters, and ectonucleotidases activity after treatment of adult zebrafish via i.p with SWCNT, as well as the expression of genes related to antioxidant response in both adult Danio rerio and embryos of the same species exposed to SWCNT. After analysis, changes in the expression of genes such as the transcription factor Nrf2 and the catalytic and regulatory subunits of the enzyme glutamate cysteine ligase in zebrafish brains treated with SWCNT or fulerol via i.p. were observed. Normalized genes were modified when embryos were treated with SWCNT. Moreover, changes in the total antioxidant capacity and increased concentrations of neurotransmitters like dopamine and serotonin were observed in brain from zebrafish treated with SWCNT. Given these results, we conclude that carbon nanomaterials can influence the neurological system of zebrafish through pronounced changes in neurotransmitters responsible for

depression and anxiety, as well as the physiological level, by regulating the expression of important genes in antioxidant defense.

Key words: nanomaterials, nanotoxicology, oxidative stress, antioxidant system, zebrafish *Danio rerio*, neurotransmitters.

3. Introdução

3.1 Nanomateriais

Os nanomateriais compreendem partículas tais como as nanoesferas de carbono (fulerenos), as nanopartículas de óxido metálico e de metal, além dos nanotubos de carbono. Os nanomateriais possuem diâmetro ou pelo menos uma dimensão menor que 100 nanômetros (Liu et al., 2013). Com apenas a redução no tamanho, as propriedades dos nanomateriais podem mudar drasticamente em relação a, por exemplo, condutividade elétrica, características magnéticas, dureza, área de superfície ativa, reatividade química e atividade biológica (Karlsson et al., 2008). Essas propriedades podem ser importantes no contexto de aplicações tecnológicas mas podem, em outro aspecto, serem indesejadas devido a possibilidade de causarem efeitos tóxicos. Dessa forma, devido ao uso crescente e rápido das nanotecnologias, é imprescindível uma investigação crítica da toxicidade potencial dos nanomateriais, uma vez que estes já estão sendo incorporados em diversos produtos, e o consequente uso deste tipo de materiais pode resultar na sua liberação e ingresso no ambiente e biota (Oberdörster, 2004; Brant et al., 2005; Mueller e Nowack, 2008; Panessa-Warren et al., 2009, Liu et al., 2013). Dessa forma, a perspectiva generalizada do uso de nanomateriais manufaturados nos produtos de consumo pode aumentar dramaticamente a exposição ambiental, ocupacional e pública.

Nos ambientes aquáticos, a toxicidade dos nanomateriais pode ser modificada devido suas várias propriedades químicas, incluindo a partição dos nanomateriais para o sedimento e matéria particulada em suspensão, degradação biológica e abiótica, aglomeração e agregação das partículas (Galloway et al. 2010). A aglomeração, por exemplo, diminui a energia

livre de interface e, com isso, a reatividade dos nanomateriais (He e Zhao, 2005).

Vários biomarcadores, os quais incluem uma variedade de medidas de respostas moleculares, celulares e fisiológicas específicas de espécies-chave para a exposição de contaminantes (Allen and Moore, 2004), podem ser usados para avaliar os efeitos dos nanomateriais, incluindo respostas antioxidantes como atividades da superóxido dismutase (SOD), glutationa peroxidase (GPx) e glutationa S-transferase (GST), níveis de glutationa (GSH), peroxidação lipídica e outros. Em trabalho realizado por Xiong et al. (2011), zebrafish (*Danio rerio)* tratados com nanopartículas de titânio ($npTiO_2$) tiveram um aumento na atividade da enzima SOD e níveis aumentados de GSH e de peroxidação lipídica no estômago, enquanto foi observada uma diminuição na atividade das enzimas SOD e catalase (CAT) no fígado do mesmo animal. Nanomateriais como $npTiO_2$ parecem de fato mediar a produção de espécies reativas de oxigênio (ERO) e modular o sistema antioxidante, como também se observou em trabalho realizado com o microcrustáceo *Daphnia magna* expostas a $npTiO_2$ que teve as atividades de CAT, GST e GPx aumentadas (Kim et al., 2010).

As GSTs são uma família de enzimas de fase II as quais detoxificam uma grande variedade de tóxicos e intermediários reativos sendo as várias isoformas de GST responsáveis por metabolizar muitos poluentes ambientais, como pesticidas, antibióticos e hidrocarbonetos aromáticos policíclicos (PAHs) e, entre as isorformas da GST, a classe pi possui alta eficiência em conjugar metabólitos carcinogênicos como benzo[a]pireno com GSH (Garner e Di Giulio, 2012). Estudos utilizando embriões de zebrafish demonstram que uma expressão aumentada da

enzima glutationa S-transferase pi 2 (GSTp2) precede as deformidades cardíacas causadas pela co-exposição de um translocador nuclear receptor aril hidrocarboneto (AHR) e de um inibidor CYP1 (Timme-Laragy et al., 2009; Van Tiem and Di Giulio, 2011).

Um dos mecanismos mais discutidos, além dos efeitos induzidos a saúde pelas partículas ambientais, é a habilidade dos nanomateriais de promover estresse oxidativo pela formação de ERO que, em excesso, podem causar danos aos componentes biológicos através da oxidação de lipídios, proteínas e DNA. *In vivo*, os nanomateriais podem ser metabolizados ou alterados (Fischer e Chan, 2007) e, devido o seu pequeno tamanho, podem entrar facilmente nos tecidos, células, organelas e estruturas biomoleculares funcionais (DNA, ribossomos, etc) já que o tamanho físico de uma nanoestrutura é similar ao de muitas moléculas, como anticorpos e proteínas, e estruturas biológicas, como os vírus (Fischer e Chan, 2007). Além disso, alguns nanomateriais prontamente são distribuídos no corpo, depositando-se em órgãos-alvo, penetrando membranas celulares e mitocôndrias, podendo provocar respostas injuriosas (Nel et al., 2006).

3.2 Nanotubos de carbono

Devido suas propriedades físicas exclusivas, bem como as características mecânicas, eletrônicas e térmicas, além de suas propriedades químicas, os nanotubos de carbono (NTC), classe de nanomateriais compostos inteiramente de carbono, têm sido intensivamente estudados. Estes nanomateriais possuem diâmetro de cerca de 1 nm, e um comprimento de 10 μm ou mais, além de uma grande

superfície relativa que pode atingir 1310 $m^2 g^{-1}$ (Mouchet et al., 2008). Esta última característica permite que os nanotubos tenham uma alta sensibilidade de detecção e reconhecimento molecular e que possam ser modificados com grupos funcionais de variadas complexidades, como moléculas de fármacos com propriedades anticancerígeno, antivirais ou antibacterianos (Cheng et al., 2009).

Vários estudos têm documentado que metais de transição utilizados na síntese dos NTC e liberados destes são os responsáveis por promover estresse oxidativo. No entanto, NTC altamente purificados também causam geração de ERO, as quais são geradas por conta da grande área superficial, (Liu et al., 2012) uma vez que os NTC poderiam ativar moléculas sinalizadoras específicas associadas com estresse oxidativo, como a proteína ativadora (AP-1), o fator nucler B (NF-B) e MAP quinases, os quais podem liberar citocinas inflamatórias atreladas a queda de antioxidantes, poli-ADP ribose polimerase-1 (PARP1), proteína quinase p38 e proteína quinase serina-treonina (Akt) (Pacurari et al., 2008; Liu et al., 2012).

Muitos trabalhos apresentam resultados conflitantes, como um alto índice de efeitos tóxicos em vários tipos celulares depois da exposição a NTC, enquanto outros demonstram poucas ou insignificantes respostas celulares após tratamento com estes nanomateriais. Esta inconsistência pode ser explicada por muitos fatores externos e intrínsecos dos NTC, tais como carga e modificação da superfície, forma, comprimento, aglomeração ou número de camadas, as quais podem influenciar a toxicidade destes nanomateriais (Liu et al, 2012; Shvedova et al., 2012). No entanto, é imprescindível, que estudos acerca da toxicidade dos NTC

continuem sendo desenvolvidos, uma vez que a incorporação dos NTC no ambiente, tanto em sua forma de parede simples (SWCNT) como de parede múltipla (MWCNT) é altamente relevante devido as diversas aplicações vislumbradas pelo uso desses, o que leva a um volume de produção substancial e, consequentemente, pode aumentar as emissões no ar, no solo e sedimentos, podendo chegar às águas subterrâneas, reservatórios e sistemas fluviais (Parks et al., 2013) (Figura 1). Nesses compartimentos ambientais, os processos físicos e químicos podem alterar suas propriedades, por exemplo, fatores abióticos como luz ultravioleta podem alterar o revestimento dos NTC (Helland et al., 2007) e com isso modificar o comportamento deles e, assim, influenciar seu destino ambiental e impacto. Além disso, os NTC são um dos materiais sintéticos menos biodegradáveis, sendo totalmente insolúveis em água na forma prístina e lipofílicos por natureza e, uma vez que químicos biopersistentes e lipofílicos podem se acumular ao longo da cadeia alimentar, os NTC poderiam eventualmente ser capturados por comunidades microbianas e raízes e, consequentemente, se acumularem em tecidos de plantas (Oberdörster et al, 2006) ou ainda serem tóxicos a células humanas, demais animais e bactérias (Jaisi et al., 2008).

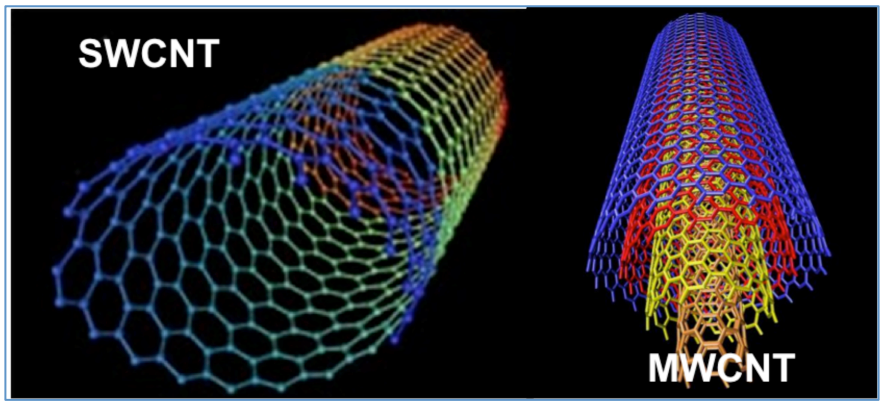

Figura 1. Esquema representativo da estrutura dos nanotubos de carbono (extraído da web)

Em trabalho realizado por Chen e colaboradores (2012), MWCT exibiram uma toxicidade aguda que levou a uma inibição na proliferação celular e sérias malformações no desenvolvimento de embriões de zebrafish que foram expostos a suspensões de 25 mg/L de NTC. Larvas com 14 dias de vida, tratadas no primeiro estágio pós fertilização com microinjeção de MWCNT, tiveram menor taxa de sobrevivência, cerca de 50%, quando comparadas ao controle, sugerindo um efeito negativo no potencial reprodutivo (Cheng et al., 2009).

3.3 Fulerol

Entre os nanomateriais, o fulereno C_{60} tem um arranjo de seis átomos de carbono em uma esfera oca de ciclopentanos e ciclohexanos que promovem uma alta estabilidade e persistência em forma de agregados coloidais em água (Johnston et al., 2010). As características estruturais e eletrônicas do fulereno C_{60} permitem que a ele sejam ligados um grande número de diferentes grupos funcionais e, a funcionalização induzida da molécula de fulereno com múltiplos grupos hidroxila (OH), produz o fulerol ($(C_{60}(OH)_n$), um derivado do fulereno com alta solubilidade a água (Britto et al., 2012) (Figura 2). Esta funcionalização de fulerenos com, por exemplo, grupos OH ou COOH, induz tais mudanças em suas propriedades físico-químicas, que além de torná-lo mais hidrossolúvel, também promove uma diminuição da citotoxicidade (Johansen et al, 2008).

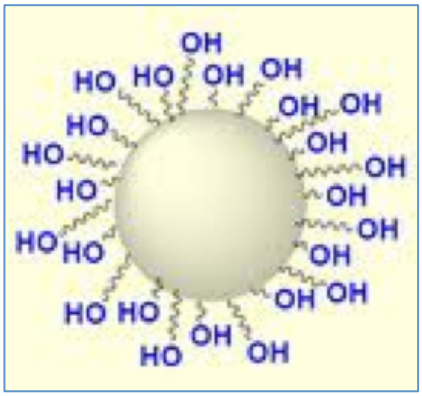

Figura 2. Esquema representativo da estrutura do fulerol (extraído da web).

No caso específico do fulereno funcionalizado com grupos OH, o fulerol, é sabidamente um antioxidante eficiente capaz de reduzir a morte neuronal mediada por ERO envolvidas com os receptores glutamato (Jin et al., 2000).

Os fulerenos polihidroxilados têm sido recentemente reconhecidos como moduladores exógenos do balanço redox, sendo capazes de exercer efeitos antioxidantes tanto em sistemas *in vitro* quanto *in vivo*. Assim, a capacidade antioxidante do fulerol é sua propriedade mais explorada. Estudos realizados *in vivo* mostraram a eficiência radioprotetiva do fulerol em ratos irradiados, diminuindo a toxicidade tecidual normal provocada pela radioterapia (Bogdanovic et al., 2008).

A disfunção mitocondrial é consequência do dano oxidativo causado por níveis aumentados de pró-oxidantes. Portanto, a diminuição na formação de oxidantes e dano oxidativo deve ser uma forma efetiva para inibir o prejuízo mitocondrial. O fulerol é capaz de atravessar a membrana celular externa e ficar localizado preferencialmente na mitocôndria (Foley et al., 2002). O efeito protetor do fulerol foi observado por Cai e colaboradores (2008) quando o pré-tratamento com o fulerol em

modelos celulares da doença de Parkinson, que tiveram a toxicidade induzida por MPP (1-metil-4-fenilpiridínio), inibiu o estresse oxidativo, incluindo a inibição de ERO, oxidação de proteínas e dano de DNA, além de aumentar o MMP (um indicador da capacidade e integridade mitocondrial) e a atividade dos complexos I e II, sugerindo dessa forma que o fulerol pode atuar como um efetivo antioxidante mitocondrial.

Além do efeito antioxidante direto sobre os radicais livres, o fulerol pode também ter um efeito protetor indireto, tal como a indução das enzimas de fase II (Gao et al., 2007). O Nrf2, fator nuclear eritróide 2 relacionado ao fator 2, é conhecido como um regulador chave da expressão de genes mediadores da resposta antioxidante como a GCL (ɣ-glutamilcisteina ligase), enzima limitante para a síntese de um dos principais antioxidantes intracelulares como é a glutationa (GSH) (Kobayashi e Iamamoto, 2005). O pré-tratamento de fulerol em células antes expostas ao 1-metil-4-fenilpiridínio (MPP) aumentou significativamente a expressão do Nrf2 total e do Nrf2 nuclear, restaurando-os para os níveis controle. O pré-tramento com fulerol também elevou a atividade da GCL e aumentou os níveis de GSH (Cai et al., 2008), sendo esses resultados consistentes com a proteção do fator de transcrição Nrf2 e, ainda, sugerindo o fulerol como uma molécula capaz de regular a expressão de genes importantes na defesa antioxidante.

Há diferentes tipos de fatores de transcrição adaptados para proteger contra o estresse oxidativo ou para gerenciar as consequências deste, como o receptor ativado por proliferadores de peroxissoma gama (PPAry), o fator de ativação de transcrição 4 (ATF4) e muitos outros (Cho

et al., 2006). Em condições normais e não estressantes, o fator de transcrição Nrf2 é sequestrado no citosol pela ação da Keap 1, proteína ligante regulatória do Nrf2, que funciona como um adaptador para proteínas que direcionam o Nrf2 para ubiquitinação para que este seja degradado por proteossomas (Osburn e Kensler, 2008). Este mecanismo de degradação proteolítica do Nrf2 é muito eficiente e, sendo assim, a meia-vida do Nrf2 é de aproximadamente 20 min, sendo difícil sua detecção em condições normais. Quando em situação de estresse, a degradação proteossomal é diminuída e há um aumento na translocação do Nrf2 para o núcleo onde esse é heterodimerizado com uma pequena proteína de fibrossarcoma músculo-aponeurótico (Maf) e liga-se aos elementos de resposta antioxidante (ARE) dando início à transcrição de genes citoprotetores (Klaassen e Reisman, 2010) (Figura 3).

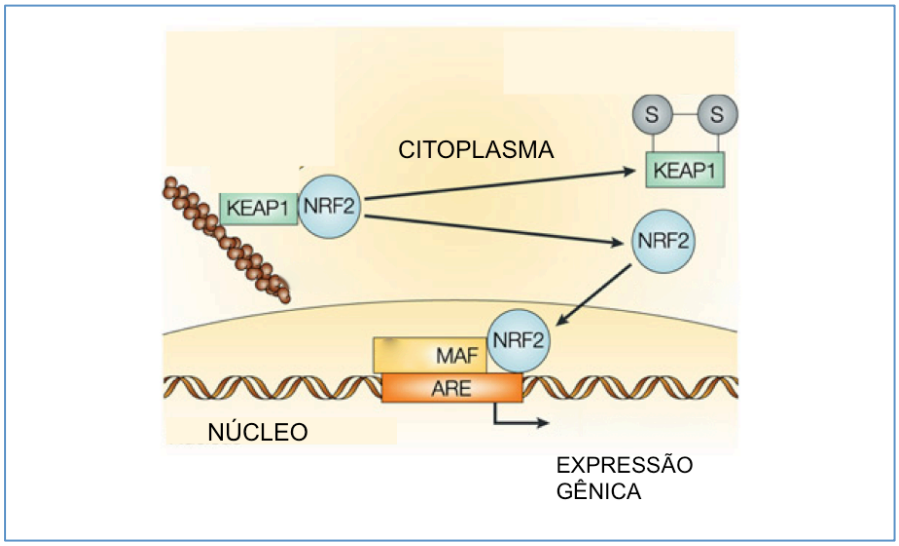

Figura 3. Esquema representativo da ação do fator de transcrição Nrf2 (extraído e modificado da web).

3.4 Estresse oxidativo e neurotoxicidade dos nanomateriais

O estresse oxidativo é uma via comum de toxicidade e doenças e um organismo pode estar sujeito a ele por meio de diversos mecanismos, como ser induzido diretamente por um agente oxidante, tal como o peróxido de hidrogênio (H_2O_2), produzido através da indução do citocromo P450 ou outros processos bioquímicos, ou por um agente externo, como os NTC, que inibem a produção de moléculas antioxidantes, tal como a GSH (Zhao et al., 2012), responsável por manter o balanço oxidativo, agindo na detoxificação de metabólitos e nas ERO associadas com a exposição química e a doenças (Usenko et al., 2008). De fato, um primeiro e rápido efeito dos NTC sobre as células é a formação de ERO, gerando estresse oxidativo, que é visto como um fator chave que afeta a funcionalidade celular (Shvedova et al., 2012).

As ERO podem alterar a sinalização redox e o controle de várias funções celulares (Jones, 2006), sendo que o equilíbrio redox intracelular pode ser perturbado pela presença de nanomateriais, os quais como dito, podem induzir o aumento da concentração intracelular de ERO. Este aumento pode ser induzido pelo próprio nanomaterial, ou indiretamente pelo distúrbio das vias de degradação de ERO. Ambos causam uma produção adicional de ERO, que interagem com membranas celulares, DNA, e/ou outros compostos celulares, danificando os componentes destas células (Helland et al., 2007).

O fator de transcrição chave na regulação da proteção antioxidante e detoxificação é o fator de transcrição Nrf2, o qual possui a habilidade de controlar diferentes aspectos da proteção nuclear, incluindo reparo de DNA, redução de quinonas e síntese de GSH (Da Rocha et al., 2013;

Maher and Yamamoto, 2010). Dessa forma, na medida em que as membranas de DNA podem ser afetadas por nanotubos, a expressão do fator transcrição Nrf2, que ativa genes citoprotetores, como os antioxidantes, também pode ser alterada. Isto foi observado num trabalho que forma parte desta tese, realizado com nanotubos de parede simples (SWCNT) injetados intraperitonealmente em zebrafish adultos, quando foi verificado um aumento na expressão do fator de transcrição Nrf2 em relação ao controle, mostrando que o SWCNT teve um comportamento pró-oxidante capaz de promover um aumento da expressão de Nrf2 no cérebro de zebrafish tratados com este nanomaterial (Da Rocha et al., 2013). É sabido que um dos fatores que determinam a migração do fator de transcrição Nrf2 do citoplasma para o núcleo é o estado redox que em situações pró-oxidantes libera este fator de transcrição da proteína Keap1, permitindo sua translocação para o núcleo (Kang et al., 2005).

Um prejuízo cerebral pode ocorrer por alterações nos níveis de neurotransmissores. Uma diminuição nos níveis de dopamina (DOPA), por exemplo, acontece em resposta a um aumento de ERO (Wang et al., 2009), o que propicia que o estresse oxidativo seja responsável pelo declínio de processos cognitivos relacionados a idade e a patogênese de doenças neurodegenerativas (Liu et al., 2013). Esse potencial risco para doenças neurodegenerativas, provocado pela depleção de DOPA, foi demonstrado em trabalho realizado por Wu e colaboradores (2011), quando foi possível observar que nanopartículas de sílica (SiO_2-NPs) têm um impacto negativo sobre o corpo estriado, bem como sobre neurônios doparminérgicos de cérebros de ratos tratados com este nanomaterial. A mesma alteração nos níveis de dopamina foi observada em cérebros de

ratos expostos a dióxido de titânio (TiO_2-NPs) (Hu et al., 2011). Da mesma maneira, trabalhos *in vitro* utilizando uma linhagem celular, PC-12, demonstraram que os níveis de DOPA também diminuíram quando estas células foram tratadas com nanopartículas de manganês (Hussain et al., 2011), bem como quando expostas a nanopartículas de cobre (Wang et al., 2009).

O sistema colinérgico também parece ser alterado quando em presença de nanomateriais, como os nanotubos de carbono, através da inibição da acetilcolinesterase, AChE (Wang et al., 2009). Esta enzima hidrolisa o neurotransmissor acetilcolina (ACh) e sua inibição causa a acumulação de ACh na fenda sináptica, interferindo no controle de respostas fisiológicas e comportamentais e, eventualmente, levando a uma falha respiratória e morte celular (Worek et al., 2002) (Figura 4).

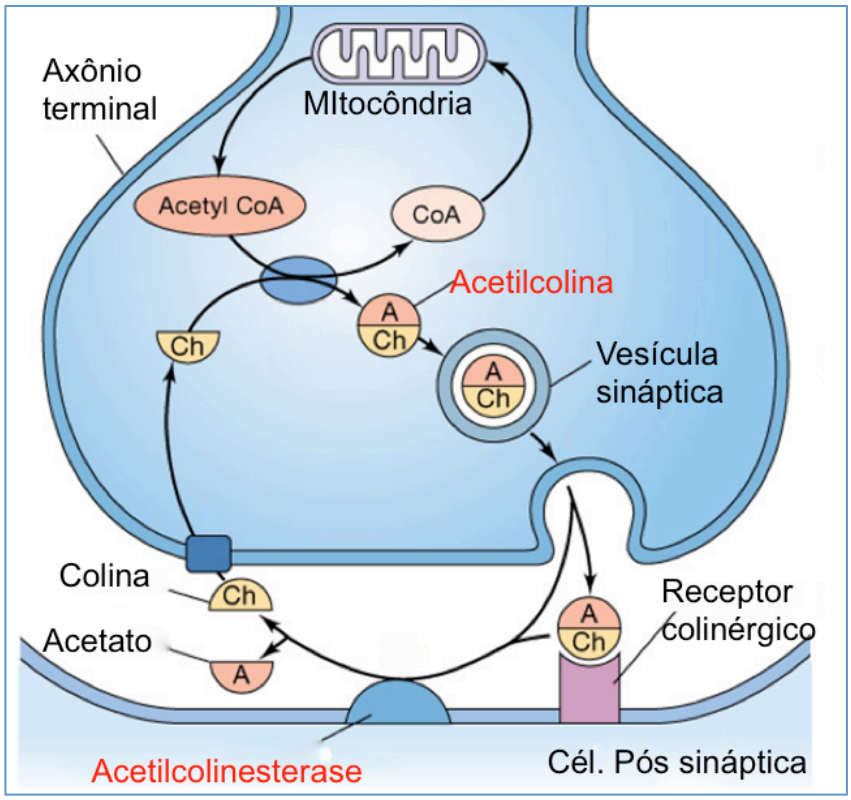

Figura 4. Esquema representativo da atividade da enzima acetilcolinesterase (extraído e modificado da web).

Em trabalho *in vitro* realizado por Wang e colaboradores (2009), a inibição de AChE por nanotubos de carbono de parede múltipla e simples teve uma relação dose-resposta, e as suas concentrações inibitórias medianas (IC_{50}) foram de 156 e 96 mg/L, respectivamente, mostrando que esses nanomateriais podem exercer neurotoxicidade através da inibição da AChE.

A exemplo dos neurotransmissores citados, o ATP também atua como um neurotransmissor e/ou neuromodulador na transmissão sinaptica (Burnstock, 2000). O ATP é um nucleotídeo trifosfato presente em todas as células e que está envolvido na regulação de muitos processos patofisiológicos no meio extracelular, podendo ser co-liberado com outros neurotransmissores como ACh, noradrenalina, serotonina (5HT) e ácido γ-aminobutírico (GABA) (Takanishi e Takeda, 1973; Burnstock, 2012).

Os nucleotídeos e nucleosídeos atuam como moléculas sinalizadoras envolvidas em uma gama de efeitos biológicos e esses nucleotídeos são degradados por hidrólise de uma cascata de enzimas chamadas ectonucleotidases, as quais estão ancoradas na membrana celular e determinam a viabilidade de ligantes, tais como o ATP, ao seu receptor específico (Zimmermann, 2011). (Figura 5).

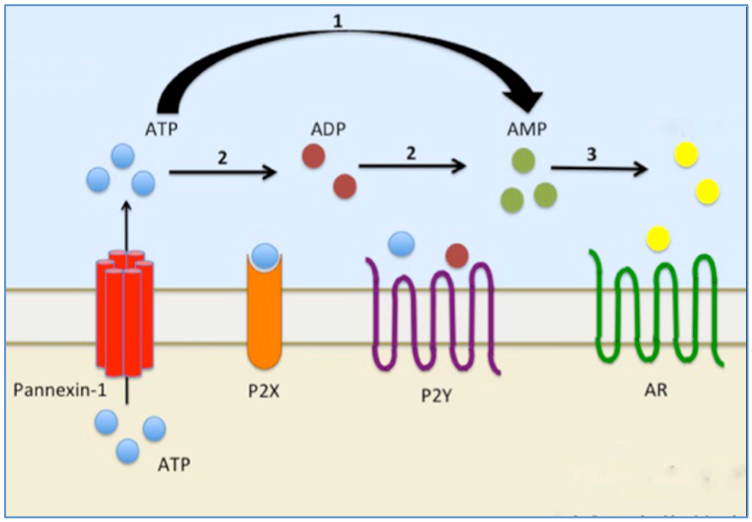

Figura 5. Esquema representativo da atividade das enzimas ectonucleotidases (extraído e modificado da web).

Dada a importância dos nucleotídeos e nucleosídeos nos processos de funcionamento cerebral, torna-se importante averiguar se nanomaterias de carbono, como nanotubos, podem ter a capacidade de alterar a atividade das ectonucleotidases responsáveis pela hidrólise daqueles.

3.5 Modelos experimentais

Ainda que os NTC sejam utilizados e produzidos, existem poucos estudos realizados *in vivo* acerca do seu potencial de toxicidade, especialmente em organismos aquáticos, e um dos mais promissores modelos de estudo é o zebrafish. Este modelo tem sido usado extensivamente para a descoberta de drogas e avaliação dos efeitos integrados e subsequente identificação dos mecanismos de ação tóxica de químicos e de nanomateriais (Usenko et al., 2007; Costa et al., 2012; Da Rocha et al., 2013; Ferreira et al., 2014). Além de ser amplamente utilizado como modelo animal em pesquisas toxicológicas e aplicações

biomédicas, também é utilizado em análises comportamentais por apresentar um paralelismo genético com humanos que inclui a barreira hemato-encefálica, células endoteliais e respostas imunogênicas (Fako e Furgeson, 2009) além de ser um modelo bastante utilizado em estudos de nanotoxicidade nos últimos anos, como na avaliação dos efeitos de nanomaterias de carbono como nanotubos (Cheng eta al., 2009; Da Rocha et al., 2013; Wang et al., 2013).

A toxicidade dos nanomateriais, tanto *in vitro* quanto *in vivo*, é atribuída a vários fatores como, por exemplo, o comprimento, o tipo de funcionalização, a concentração, a duração da exposição, aos métodos de exposição e ao dispersante usado para solubilizar os nanotubos (Firme III e Bandaru, 2010). Embora os resultados de alguns trabalhos aumentem a preocupação acerca da toxicidade dos NTC como, por exemplo, a alta inibição de proliferação celular e malformações no desenvolvimento de embriões de zebrafish causada por toxicidade aguda de nanotubos de carbono de parede múltipla (MWCNT) (Chen et al., 2012) e a diminuição da capacidade antioxidante total em cérebros de zebrafish tratados com injeção intraperitoneal de SWCNT (Da Rocha et al., 2013), não há, ainda, nenhuma investigação sistemática *in vivo* sobre os efeitos toxicológicos nos NTC administrados via microinjeção em embriões de zebrafish ou, ainda, qualquer trabalho que aponte o comportamento de neurotransmissores e ectonucleotidases em cérebros oriundos de zebrafish tratados com SWCNT via intraperitoneal.

4. Objetivos

4.1 Objetivo geral

Investigar os efeitos da capacidade antioxidante, dano oxidativo, expressão gênica e neurotransmissores em zebrafish *Danio rerio* expostos a nanotubos de carbono de parede simples (SWCNT) ou fulerol

4.2 Objetivos específicos

(a) Investigar os efeitos dos SWCNT e fulerol em cérebros oriundos de zebrafish adultos tratados via injeção intraperitoneal (i.p.), em relação a peroxidação lipídica (TBARS) e capacidade antioxidade total contra peróxi-radicais (ACAP);

(b) Verificar a expressão de genes relacionados a resposta antioxidante como o fator de transcrição Nrf2 e as subunidades catalíticas e regulatórias da enzima glutationa cisteína ligase (GCL) por transcriptase reversa (RT-PCR) e expressão gênica da enzima acetilcolinesterase (AChE) em cérebros retirados de zebrafish adultos tratados via i.p. com SWCNT e fulerol;

(c) Determinar a atividade da AChE, a concentração dos neurotransmissores dopamina (DOPA) e serotonina (5HT) e a atividade de enzimas ectonucleotidases em cérebros oriundos de zebrafish adultos tratados via i.p. com SWCNT;

(d) Investigar os efeitos dos SWCNT sobre os embriões de zebrafish, em relação a expressão de genes relacionados a resposta antioxidante, como GCLc e glutationa peroxidase 1 (GPX1);

(e) verificar a expressão de genes normalizadores em embriões de zebrafish quando estes são expostos a SWCNT.

5. Referências Bibliográficas

Allen, J.I., Moore, M.N. 2004. Environmental prognostics: Is the current use of biomarkers appropriate for environmental risk evaluation? Marine Environmental Research 58: 227-232.

Bogdanovic, V., Stancov, K., Icevic, I., Zikic, D., Nikolic, A., Solajikc, S., Dejordjevic, A., Bogdanovic, G. 2008. Fullerenol $C_{60}(OH)_{24}$ effects antioxidative enzymes activity in irradiated human erythroleukemia cell line. Journal of Radiation Research 49: 321-327.

Burnstock, G. 2000. Potencial therapeutic target in the rapidly expanding field of purinergic signaling. Clinical Medicine 2: 45-53.

Burnstock, G. 2012. Purinergic signaling: Its unpopular beginning, its acceptance and its exciting future. Bioessays 34: 218-225.

Brant, J., Lecoanet, H., Wiesner, M.R. 2005. Aggregation and deposition characteristics of fullerene nanoparticles systems. Journal of Nanoparticle Research 7: 545-553.

Britto, R.S., Garcia, M.L., da Rocha, A.M., Flores, J.A., Pinheiro, M.VB., Monserrat, J.M., Ferreira, J.L.R. 2012. Effects of carbono nanomaterials fullerene C_{60} and fullerol $C_{60}(OH)_{18-22}$ on gills of fish *Cyprinus carpio* (Cyprinidae) exposed to ultraviolet radiation. Aquatic Toxicology 114: 80-87.

Cai, X., Hao, J., Zhang, X., Yu, B., Ren, J., Luo, C., Li, Q., Huang, Q., Shi, X., Li, W., Liu, J. 2010. The polyhydroxylated fullerene derivative $C_{60}(OH)_{24}$ protects mice from ionizing-radiation-induced immune and mitochondrial dysfunction. Toxicology and Pharmacology 243: 27-34.

Chen, L.Q., Hu, P.P., Zhang, L., Huang, S., Luo, L.F., Huang, C.Z. 2012. Toxicicity of graphene oxide and multi-walled carbono nanotubes against human cells and zebrafish. Science China 10: 2209-2216.

Cheng, J., Chan, C.M., Veca, L.M., Poon, W.L., Chan, P.K., Qu, L., Sun, Y.P., Cheng, S.H. 2009. Acute and long-term effects after single loading of functionalized multi-walled carbon nanotubes into zebrafish (*Danio rerio*). Toxicology and Applied Pharmacology, 235: 216-225.

Cho, H.Y., Reddy, S.P., Kleeberger, S.R. 2006. Nrf2 defends the lung from oxidative stress. Antioxidants & Redox Signaling 8: 76-87.

Da Rocha, A.M., Ribas Ferreira, J., Marti Barros, D., Pereira, T.C.B., Bogo, M.R., Oliveira, S., Geraldo, V., Lacerda, R.G., Ferlauto , A.S., Ladeira, L.O., Pinheiro, M.V.B., Moserrat, J.M. 2013. Gene expression. And biochemical responses in brain of zebrafish *Danio rerio* exposed to organic nanomaterials: carbon nanotubes (SWCNT) and fullerenol ($C60(OH)_{18–22}(OK_4)$). Comparative Biochemistry and Physioloy Part A, 165: 460-467.

Fako, V.E., Furgeson, D.Y. 2009. Zebrafish as a correlative and predictive model for assessing biomaterial nanotoxicity. Advanced Drug Delivery Reviews 61: 478-486.

Firme III, C.P., Bandaru, P.R. 2010. Toxicity issues in the application of carbon nanotubes to biological systems. Nanomedicine: Nanotechnology, Biology and Medicine 6: 245-256.

Fischer, H.C., Chan, W. 2007. Nanotoxicity: The growing need for *in vivo* study. Current Opinion in Biotechnology 18: 565-571.

Foley, S., Crowley, C., Smaihi, M., Bonfils, C., Erlanger, B.F., Seta, P., Larroque, C. 2002. Cellular localization of a water-soluble fullerene

derivative. Biochemical and Biophysical Research Communication 294: 116-119.

Galloway, T., Lewis, C., Dolciotti, I., Johnston, B.D., Moger, J., Regoli, F. 2010. Sublethal toxicity of nano-titanium dioxide and carbon nanotubes in a sediment dwelling marine polychaete. Environmental Pollution 158: 1748-1755.

Gao, J., Zhu, Z.R., Ding, H.Q., Qian, Z., Zhu, L., Ke, Y. 2007. Vulnerability of neurons with mitochondrial dysfunction to oxidative stress is associated with down-regulation of thioredoxin. Neurochemistry International 50: 379-385.

Garner, V.T., Di Giulio, T.T. 2012. Glutathione transferase pi class 2 (GSTp2) protects against the cardiac deformities caused by exposure to PAHs but not PCB-126 in zebrafish embryos. Comparative Biochemistry and Physilogy, Part C 155: 573-579.

He, F., Zhao, D. 2005. Preparation and characterization of a new class of starch stabilized bimetallic nanoparticles for degradation of chlorinated hydrocarbons. Environmental Science and Technology 39: 3314-3320.

Helland, A., Wick, P., Koehler, A., Schmid, K., Som, C. 2007. Reviewing the environmental and human health knowledge base of carbon nanotubes. Environmental Health Perspectives 115: 1125-1131.

Hussain, S.M., Javorina, A.K., Schrand, A.M., Duhart, H.M., Ali, S.F., Schlager, J.J. 2006. The interaction of manganese nanoparticles with PC-12 cells induces dopamine depletion. Toxicological Sciences 92: 456-463.

Jaisi, D.P., Saleh, N.B., Blake, R.E., Elimelech, M. 2008. Transport of single-walled carbon nanotubes in porous media: Filtration mechanisms and reversibility. Environmental Science and Technology 42: 8317-8323.

Jin, H., Chen, W.Q., Tang, X.W, Chiang, L.Y., Scholoss, J.V., Wu, J.Y. 2000. Polyhydroxylated C_{60}, fullerenols, as glutamate receptor antagonists and neuroprotective agents. Journal of Neuroscience Research 62: 600-607.

Johansen, A., Pedersen, A.L., Jensen, K.A., Karlson, U. 2008. Effects of C_{60} fullerene nanoparticles on soil bacteria and protozoans. Environmental Toxicology and Chemistry 27: 1895-1903.

Johnston, H.J., Hutchison, G.R., Christensen, F.M., Aschberger, K., Stone, V. 2010. The biological mechanisms and physicochemical characteristics responsible for driving fullerene toxicity. Toxicological Sciences 114: 162-182.

Jones, D.P. 2006. Disruption of mitochondrial redox circuitry in oxidative stress. Chemico-Biological Interactions 163: 38-53.

Kang, K.W., Lee, S.J., Kim, S.G. 2005. Molecular mechanism of Nrf2 activation by oxidative stress. Antioxidants & Redox Signaling 7, numbers 11 & 12.

Karlsson, H., Cronholm, P., Gustafsson, J., Moller, L. 2008. Copper oxide nanoparticle are highly toxic: A comparison between metal oxide nanoparticles and carbon nanotubes. Chemical and Research and Toxicology 21: 1726-1732.

Kim, K.T., Klaine, S.J., Cho, L., Kim, S.H., Kim, S.D. 2010. Oxidative stress responses of *Daphnia magna* exposed to TiO_2 nanoparticles according to size fraction. Science of the Total Environment 408: 2268-2272.

Klaassen, C.D., Reisman, S.A. 2010. Nrf2 the rescue: Effects of the antioxidant/eletrophilic response on the liver. Toxicology and Applied Pharmacology 244: 57-63.

Kobayashi, M., Yamamoto, M. 2005. Molecular mechanisms activiting the Nrf2-Keap1 pathway of antioxidant gene regulation. Antioxidants & Redox Signaling 7: 385-394.

Liu, A., Sun, K., Yang, J., Zhao, D. 2008. Toxicological effects of multi-wall carbon nanotubes in rats. Journal of Nanoparticles Research 10: 1303-1307.

Liu, Y., Zhao, Y., Sun, B., Chen, C. 2012. Understanding the toxicity of carbon nanotubes. Accounts of Chemical Research 46: 702-713.

Liu, P., Huang, Z., GU, N. 2013. Exposure to silver nanoparticle does not affect cognitive outcome or hippocampal neurogenesis in adult mice. Ecotoxicology and Envirnmental Safety 87: 124-130.

Maher, J., Yamamoto, M. 2010. The rise of antioxidante signaling- The evolution and hormetic actions of Nrf2. Toxicology and Applied Pharmacology 244: 4-15.

Mouchet, F., Landois, P., Sarremejean, E., Bernard, G., Puech, P., Pinelli, E., Flahaut, E., Gauthier, L. 2008. Characterization an in vivo ecotoxicity evaluation of double-wall carbon nanotubes in larvae of the amphibian *Xenopus laevis*. Aquatic Toxicology 87: 127-137.

Mueller, N., Nowack, B. 2008. Exposure modeling of engineered nanoparticles in the environment. Environmental Science and Technology 42: 4447-4453.

Oberdörster, E. 2004. Manufactured nanomaterials (fullerenes, C_{60}) induce oxidative stress in brain of juvenile largemouth bass. Environmental Health Perspectives 112: 1058-1062.

Oberdörster, E., Zhu, S., Blickey, T.M., McClellan-Green, P.,Haasch, M.L. 2006. Ecotoxicology of carbon-based engineered nanoparticles: effects of fullerene (C_{60}) on aquatic organisms. Carbon 44: 1112-1120.

Osburn, W.O., Kensler, T.W. 2008. Nrf2 signaling: An adaptive response pathway for protection against environmental toxic insults. Mutation Research 659: 31-39.

Pacurari, M., Yin, X., Zhao, J., Ding, M., Leonardo, S., Schwegler-Berry, D., Ducatman, B., Sbarra, D., Hoover, M., Castranova, V., Vallyathan, V. 2008. Raw single-wall carbono nanotubes induce oxidative stress ans active Mapks, AP-1, NF-Kappa b, and Akt in normal and malignant human mesothelial cells. Environmental Health Perspectives 116: 1211-1217.

Panessa-Warren, B.J., Maye, M.M., Warren, J.B., Crosson, K.M. 2009. Single walled carbon nanotube reactivity and cytotoxicity following extended aqueous exposure. Environmental Pollution 157: 1140-1151.

Parks, A.N., Portis, L.M., Schiers, P.A., Washburn, K.M., Perron, M.M. Burgess, R.M., Ho, L.T., Chandler, G.T., Ferguson, P.L. 2013. Bioaccumulation and toxicity of single-walled carbono nanotubes to benthic organisms at the base of the marine food chain. Environmental Toxicology and Chemistry 32: 1270-1277.

Senger, M.R., Rico, E.P., Arizi, M.B., Rosemberg, D.B., Dias, R.D., Bogo, M.R., Bonan, C.D. 2005. Carbofuran and malathion inhibit nucleotide hydrolysis in zebrafish (*Danio rerio*) brain membranes. Toxicology 112, 107-115.

Shvedova, A.A., Pietroiusti, A., Fadeel, B., Kagan, V.E. 2012. Mechanisms of carbon nanotube-induced toxicity: Focus on oxidative stress. Toxicology and Applied Pharmacology 261: 121-133.

Timme-Laragy, A.R., Van Tiem, L.A., Linney, E.A., Di Giulio, R.T. 2009. Antioxidant responses and NRF2 in synergistic developmental toxicity of PAHs in zebrafish. Toxicological Sciences 109: 217-227.

Usenko, C.Y., Harper, S.L., Tanguay, R.L. 2007. *In vivo* evaluation of carbon fullerene toxicity using embryonic zebrafish. Carbon 45: 1891-1898.

Usenko. C.Y., Stacey, L.,Tanguay, R.L. 2008. Fullerene C_{60} exposure elicits an oxidative stress response in embryonic zebrafish. Toxicology and Applied Pharmacology 229: 44-55.

Van Tiem, L.A., Di Giulio, R.T. 2011. AHR2 knockdown prevents PAH-mediated cardiac toxicity and XRE- and ARE-associated gene induction in zebrafish (*Danio rerio*). Toxicology and Applied Pharmacology 254: 280-287.

Wang, J., Rahman, M.F., Duhart, H.M., Newport, G.D., Patterson, T.A., Murdock, R.C., Hussain, S.M., Schlager, J.J., Ali, S.F. 2009. Expression changes of dopaminergic system-related genes in PC12 cells induced by manganese, silver, or copper nanoparticles. Neurotoxicology 30: 926-933.

Wang, K., Ma, J., He, M., Gao, G., Xu, H., Sang, J., Wang, Y., Zhao, B., Cui, D. 2013. Toxicity assessments of near-infrared upconversion luminescente LaF3:Yb,Er in early development os zebrafish embryos. Theranostics 3: 258-266.

Wang, Z., Zhao, J., Li, F., Gao, D., Xing, B. 2009. Adsortion and inhibition of acetylcholinesterase by different nanoparticles. Chemosphere 77: 67-73.

Worek, F., Reiter, E.G., Eyer, E.P., Szinics, E.L. 2002. Reactivation kinetics of acetylcholinesterase from different species inhibited by highly toxic organophosphates. Archives of Toxicology 76, 523-529.

Wu, J., Wang, C., Sun, J., Xue, Y. 2011. Neurotoxicity of silica nanoparticles: Brain localization and dopaminergic neurons damage pathways. ACS Nano 6: 4476-4489.

Xiong, D., Fang, T., Yu, L., Sima, Y., Zhu, W. 2011. Effects of nano-scale TiO_2, ZnO and their bulk counterparts on zebrafish: Acute toxicity, oxidative stress and oxidative damage. Science of the Total Environment 409: 1444-1452.

Zhao, X., Liu, R. 2012. Recent progress and perspectives on the toxicity of carbon nanotubes at organism, organ, cell and biomacromolecule levels. Environmental International 40: 244-256.

6. Artigo 1

Gene expression and biochemical responses in brain of zebrafish Danio rerio exposed to organic nanomaterials: Carbon nanotubes (SWCNT) and fullerenol ($C_{60}(OH)_{18–22}(OK_4)$)

(publicado na revista Comparative Biochemistry and Physiology, Part A)

7. Artigo 2

Analysis of neurotransmitters parameters in zebrafish exposed to carbon nanotubes

(a ser submetido à revista Aquatic Toxicology)

ANALYSIS OF NEUROTRANSMITTERS PARAMETERS IN ZEBRAFISH EXPOSED TO CARBON NANOTUBES

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Abstract

Given the increasing use of carbon nanotubes (CNT) in several industries, it is essential to perform *in vivo* toxicological studies with these nanomaterials in order to evaluate their potential toxicity. Dopamine (DOPA) and serotonin (5HT), along with other neurotransmitters, have an important contribution on brain functions. Brain samples from zebrafish *Danio rerio* treated i.p. with single wall CNT (SWCNT) were used to perform the determination of DOPA and 5HT besides analyzing acetylcholinesterase (AChE) and ectonucleotidases activity, lipid damage and total antioxidant activity. Results showed that treatment with SWCNT increased between 3 and 6 fold ($p<0.05$) the concentration of DOPA and 5HT. Similarly, significant reduction ($p>0.05$) in AChE activity was observed in brains of SWCNT exposed zebrafish when compared to the control groups, showing evidences of induced neurotoxicity by single walled carbon nanotubes. It can be concluded that SWCNT promote toxicity to zebrafish brain through of changes in important neurotransmitters responsible by thermoregulation, nutrition, depression and anxiety.

Key-words: nanotoxicology, nanomaterials, zebrafish, neurotransmitters, neurotoxicity

1. Introduction

Carbon nanotubes (CNT) have unique physical, chemical, electrical and mechanical properties that offer many potential applications and because their properties, a large-scale production of CNT is increasing. Under this context it is expected that fauna, flora and human will be inevitably more 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, since the human populations also increase every year (Kunzmann et al., 2011). However, it is essential to realize assays *in vivo* 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). In addition, it seems that impurities derived from catalysis and not CNT themselves are responsible for the toxicity (Ciofani et al., 2010).

The nanomaterials, as carbon nanotubes, have the ability to generate oxidative stress (Shvedova et al., 2012) and this event may be caused directly by CNT-induced reactive oxygen species (ROS) that are close or inside the cell or it may be appear indirectly due the effects of internalized CNT on mitochondrial respiration or even by a depletion of antioxidants inside the cell (Manke et al., 2013). Nanomaterials have unique properties in their translocation to the systemic circulation and central nervous system (CNS) due to their small size and large surface area (Wang et al., 2009). Only small

lipophilic molecules of less than 500 Da are able to across blood-brain barrier, including amino acids, hexoses, neuropeptides and proteins, which are transported into the brain via specific carriers (Wohlfort et al., 2012). Nanomaterials, as carbon nanotubes, posses the ability in crossing the blood-brain barrier, being able to reach the brain and cause damage (Yang et al., 2007; Wu et al., 2011).

In a study with a human epithelial cell line A549, incubation with 100 µg/ml MWCNT exert adverse effects as a decrease on metabolic activity without being internalized by human epithelial or oxidative stress (Tabet et al., 2009). However, one potentially relevant mechanism of damage is constituted by the 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).

Brain damage can be caused by depletion of neurotransmitter dopamine (DOPA) in response to an increase of reactive oxygen species (ROS) (Wang et al., 2009), generating oxidative stress which has been implicated in age-related decline in cognitive process and in the pathogenesis of many neurodegenerative diseases such as Alzheimer, Parkinson and Huntington (Liu et al., 2013).

Similarly, the cholinergic system also appears be altered when in presence of single walled carbon nanotubes (SWCNT) and multi walled carbon nanotubes (MWCNT), being reported acetylcholinesterase (AChE) inhibition (Wang et al., 2009). This enzyme hydrolyzes the neurotransmitter acetylcholine (ACh), being one of the most crucial enzymes for nerve response and function. Its

inhibition causes the accumulation of ACh, interfering in the control of a large proportion of physiological and behavioral responses in the animal and eventually leading to respiratory failure and death (Worek et al., 2002). On the other hand, inhibition of AChE may be interesting to be used in conditions in which there is significant neuronal loss of cholinergic neurons such as Alzheimer's disease.

To the best of our knowledge, it is uncertain if CNT damage is caused directly by carbon nanotubes that really cross the blood-brain barrier or if any changes occur indirectly through carbon nanotubes that reach the bloodstream and cause some injury in a given tissue and then interfering with the brain. However, in one way or another, CNT are able to promote some brain deleterious responses, as seen in recent study from our group, in which SWCNT injected i.p. in adult zebrafish were able to induce the expression of the transcription nuclear factor erytroid 2-related factor 2 (Nrf2) when compared to the control group (da Rocha et al., 2013). Nrf2 is known to be a key regulator of the expression of genes mediators of the antioxidant response like the regulatory and catalytic subunits of glutamate cysteine ligase (GCL), the limiting enzyme for the synthesis of the main intracellular antioxidant, reduced glutathione (GSH) (White et al., 2003; da Rocha et al., 2013). According to Manke et al (2013), cells and tissues respond to increasing levels of oxidative stress via antioxidant enzyme systems upon nanomaterials exposure.

The purinergic signaling system includes ATP, a triphosphate nucleotide that exists in all cells and is involved in the regulation of many pathophysiological processes in the extracellular medium. ATP can be co-released with many

neurotransmitters such as acetylcholine (ACh), glutamate, noradrenaline, serotonin (5HT) and γ-amino butyric acid (GABA) (Nakanishi e Takeda, 1973; Burnstock, 2012).

Nucleotides as ATP are ubiquitous extracellular signaling molecule, which induce many biological effects and the extracellular ATP has an important role in synaptic transmission, acting as a neurotransmitter and/or a neuromodulator (Burnstock, 2000; Senger et al., 2005). Indeed ATP can be released by exocytosis, via transmembrane channels, via transporters or through damaged membranes. Therefore, ATP appears to be one of the most omnipresent of all known extracellular signaling molecules and it can be released in the pre and postsynaptic terminal, as part of a physiological mechanism. Its release into the synaptic cleft is calcium-dependent, being stored in presynaptic vesicles and released after depolarization, acting on specific receptors (Baldissareli et al., 2012), or in response to cellular damage, such as hypoxia and injuries (Burnstock, 2012). Purinergic nucleotides are degraded by hydrolysis of a cascade consisting of a variety of enzymes called ectonucleotidases, which are anchored in the cell membrane, then determining the availability of ligands such as ATP to their specific receptors (Zimmermann, 2011).

Biochemical events, including oxidative stress, are critical mechanisms, which can cause toxicities after the exposition to nanomaterials such as inhibition of neurotransmitters like DOPA. In this study, it was evaluated zebrafish dopaminergic, serotonergic, cholinergic and purinergic systems treated with SWCNT by analyzing dopamine and serotonin levels, acetylcholinesterase activity, and ectonucleotidases activity, respectively. Additionally, it was also

evaluated antioxidant capacity and lipid peroxidation in zebrafish brain treated with SWCNT.

2. Materials and methods

2.1. Fish care

All procedures are in accordance and were approved by Ethics Committee on Animal Use (CEUA) of 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 100 fish were placed in each of the tanks of 60 L. The pH and water temperature were fixed in 7.5-8.0 and 28 °C, respectively. Thank maintenance includes a 1/3 water change three times a week after removal of the excess of food or fish waste from the bottom of the tanks using a siphon.

2.2. Synthesis and characterization of SWCNT

The production of nanotubes was performed in an electric arc chamber using Loraine carbon graphite electrodes outer diameter of 10 mm, central role of 6 mm and 8 mm depth filled with powder catalyst com- position of 4.2% Ni, 1% Y2O3, 1% FeS and 93.8% of carbon powder. The arc discharge method

consisted in the application of a high intensity electric current of 150–200 A, a tension of 17–20 V and the pressure of gas in the chamber was of 40 torr of helium.

For morphological characterization of carbon-based materials the Raman spectroscopy has been the most widely used technique in the past 20 years. In this study, SWCNT were characterized by Raman spectroscopy and for this analyzes, equipment Raman spectroscopy, Renishaw Model inVia Spectrometer was used. 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 solutions and suspensions

Purified SWCNT were prepared by acid treatment to remove metal contaminants employed as catalysts for nanotubes synthesis. SWCNT were added to a solution of pure nitric acid and sulfuric acid 99% (3:1 v/v) and the mixture was sonicated during 6 h (da Rocha et al., 2013). After, samples were centrifuged at 3,000 x g during 20 min. The pellet was washed with Milli Q water and centrifuged again. This procedure was repeated five times. Finally, the pellet was dried during two days in an oven at 45 °C (Chen et al., 2004; da Rocha et al., 2013). SWCNT suspensions were prepared through sonication 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). A dose of 30 mg/kg of fish was chosen for both SWCNT considering a previous study from our group (da Rocha et al., 2013).

2.4. SWCNT exposure

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

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 or during the experiment. After dissection, pools of five fish brains were used to compose one sample (n = 5 pools) for each measurement and then the brain samples were stored at -80°C.

All enzyme essays, lipid peroxidation and total antioxidant capacity essay were performed in at least three different experiments, each one performed in triplicate.

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 Mg^{2+} (5 mM) (da Rocha et al., 2009). The homogenates were centrifuged at 10,000 x 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 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 (Amado et al. 2009).

2.6. 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 (namoles/mg of wet tissue) was calculated employing tetramethoxypropane as a standard.

2.7. Dopamine and serotonin level

The levels of 5HT and DOPA in samples were analyzed by a high performance liquid chromatography system composed by two pumps (LC-10AD, Shimadzu, Kyoto, Japan), a communicator bus module (CBM- 10A, Shimadzu) and a Decade amperometric electro- chemical detector (Antec, The Netherlands). Brains from zebrafish i.p. injected with SWCNT were homogeinized (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 (5,000 x g) for 15 min, at 4 °C. The supernatant fraction was filtered and injected (20 ll) directly to the HPLC column (Supelcosil LC18, 150 · 4.6 mm, 5 lm particle size, Supelco). The mobile phase was isocratically pumped at a flow rate of 1 ml/min. Voltamograms of 5HT and DOPA were constructed to verify the best signals of these compounds, through injections of 5 pmol of 5HT and DOPA authentic standards. Levels of DOPA and 5HT in the samples were calculated based on standard calibration curves, which were constructed by injections of 1–20 pmol according to concentrations of 5HT and DOPA in the samples.

2.8. 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 during 10 min at 25 °C followed by starting of reaction with addition of substrate. The hydrolysis of substrate was monitored by the formation 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 (Rico et al., 2003; Senger et al., 2006). 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 $CaCl_2$ (for the NTPDase activity) or 50 mM Tris-HCl (pH 7.2) and 5 mM $MgCl_2$ (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 and the samples were kept on ice during 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 and 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. In order 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 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, significance level was fixed in 5 %.

3. Results

The presence of the characteristic bands of CNT is evidenced in Raman spectra shown in Figure 1. In the spectral region, tree 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.

Lipid peroxides levels (Fig. 2a) showed no statistically significant difference ($p > 0.05$) amongst the treated groups. However, the total antioxidant capacity against peroxi-radicals was statistically significant higher in brains from zebrafish treated i.p. with SWCNT when compared with brains coming from zebrafish of control group ($p > 0.05$) (Fig. 2b).

Fig 3. shows significantly increased levels of 5HT and DOPA in brains of zebrafish treated with 30 mg of SWCNT/kg compared with controls group (benzocaine and SDS treatments) after 48 h exposure ($p< 0.05$). Both 5HT and DOPA basal levels were some 3 to 6-fold greater in brain treated with SWCNT than in controls (Fig. 3a and b, respectively).

It was observed that AChE activity decreased in brains from zebrafish treated with SWCNT when compared with control group ($p < 0.05$) (Fig. 4).

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

4. Discussion

SWCNT can be released from products by abrasion, normal wear and tear, aging, improper use, disposal/recycling, spills, landfill leachate, industrial

effluent, waste incineration, waste water treatment, and atmospheric emissions (Parks et al, 2013) and these routs of release would likely lead to accumulation of SWCNT in terrestrial and aquatic media, and food chains. The intrinsic toxicity of 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, 2011). This reactivity is correlated with the ability of nanomaterials like SWCNT to trigger the generation of ROS that can promote oxidative stress.

Lipid peroxidation products are among the most common types of oxygenated molecules implicated in oxidative stress responses (Shevdova et al., 2012). However, in this study we did not observe lipid peroxides levels increased among the brain from zebrafish treated with SWCNT and the control. On the other hand, total antioxidant capacity against peroxi-radicals was statistically significant higher in brain from zebrafish treated with SWCNT than in controls. In this way, it is possible that the antioxidant response should cope with a pro-oxidant condition, 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 brain from zebrafish treated with SWCNT was also observed (da Rocha et al., 2013).

Neurotransmitters such dopamine (DOPA), serotonin (5HT) and acetylcholine (ACh) are essential regulators of brain functions (Nilsson, 1990; Almeida et al., 2003). The alteration of neurotransmitters and their metabolites has been used as an indicator of toxicity in the central nervous system (CNS) (Wang et al., 2009; Powers et al., 2011) and obtained results showed the influence of

SWCNT on dopaminergic, serotonergic and cholinergic system in zebrafish.

As in the case of increased DOPA and 5HT levels found in this study, intranasal instilled copper nanoparticles (Cu NPs) in mice increased DOPA levels in the striatum, cerebral cortex and cerebellum at the highest dose (40 mg/kg body weight), and the hippocampus at the lowest and medium (10 and 40 mg/kg body weight, respectively). Also, the intranasal instilled Cu NPs stimulated 5HT secretion in hippocampus and cerebral cortex of medium dose group and striatum of lowest dose (Zhang et al., 2012). In other study with mice, significantly increases in the amount of DOPA and its metabolites were observed in the striatum and prefrontal area of the TiO_2 exposed group compared to the control animals (Takahashi et al., 2010).

Acetycholinesterase (AChE) controls a large proportion of physiological and behavioral responses in the animal; thus, any changes to these regulatory abilities could be potentially harmful to fish. SWCNT and MWCNT (multi walled carbon nanotubes) had high affinity for AChE, causing 76-88% inhibition of AChE as observed in an *in vitro* study (Wang et al., 2009). Recent evidences have shown that pesticides and insecticides, metals, organochlorines and herbicides also inhibit AChE activity (for a review see Monserrat et al., 2007).

Studies suggest that pesticides can interact directly with cholinergic receptors below the concentration that inhibit AChE, affecting the second messenger systems (Bretaud et al., 2000; Senger et al., 2005). AChE inhibition can cause Ach accumulation, which is less hydrolyzed in synapses 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).

The study 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 it was not observed oxidative stress by lipid damage, an increase of antioxidant capacity was registered and these findings were surprising because the 'normal' increase in the antioxidant system is in response to an ongoing oxidative stress. Under anoxia there is no ROS generation and thus the increase in total antioxidants activity, like SOD, was possibly an anticipatory response to the oncoming stress of re-oxygenation (Hermes-Lima and Zenteno-Savin, 2002).

A problem for the vertebrate brain in anoxia is to maintain an energy production enough to preserve and satisfy the ATP consumption of the sodium and potassium pumps, given that neurotransmitters dopamine and serotonin demand molecular oxygen for their synthesis (Nilsson, 1990). As the 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 CNS (Nakanishi and Takeda, 1973), it was considered important to analyze the ectonucleotidases activity.

The purinergic signaling is a common route of cell-cell communication

involved in many neuronal and non-neuronal mechanisms and events of short and long term, including immune responses, inflammation, pain, platelet aggregation, vasodilation, proliferation and cell death. In this study, we did not observe any effect about the activity of ectonucleotidases enzymes.

5. Conclusion

In present study SWCNT did not changed ectonucletidase activities in zebrafish brain membranes, and hence did not regulate the purinergic system. In the other hand, cholinergic, serotonergic and dopaminergic system, through the neurotransmitters Ach, serotonin and dopamine, respectively was affected by SWCNT in zebrafish brain.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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References

Almeida, E.A., Bainy, A.D., Medeiros, M.H.G., Mascio, P.D, 2003. Effects of trace metal and exposure to air on serotonin and dopamine levels in tisues of the mussel *Perna perna.* Marine Pol. Bul. 46, 1485-1490.

Amado, L.L., Longaray Garcia, M., Ramos, P.B., Freitas, R.F., Zafalon, B., Ribas Ferreira, J.L., Yunes, J.S., Monserrat, J.M., 2009. A method to measure total antioxidant capacity against peroxyl radicals in aquatic organisms: Application to evaluate microcystins toxicity. Sci .Total Environ. 407, 2115-2123.

Baldissareli, L.A., Capiotti, K.M., Bogo, M.R., Ghisleni, G., Bonan, C.D., 2012. Arsenic alters behavioral parameters and brain ectonucleotidases activities in zebrafish (*Danio rerio*). Comp. Biochem. Physiol. C 155, 566-572.

Bretaud, S., Toutant, J.P., Saglio, P., 2000. Effects of carbofuran, diuron and nicosulfuron on acetylcholinesterase activity in gold fish (*Carassius auratus*). Ecotox. Environ. Safety.47, 117-124.

Burnstock, G., 2012. Purinergic signaling: Its unpopular beginning, its acceptance and its exciting future. Bioessays 34, 218-225.

Ciofani, G., Raffa, V., Vittorio, O., Cuschieri, A., Pizzorusso, T., Costa, M., Bardi, G., 2010. *In vitro* and *in vivo* biocompatibility testing of functionalized carbon nanotubes. Methods in Molecular Biology, Carbon Nanotubes: Methods and Protocols, pp. 109-119.

Chen, C.M., Chen, M., Liu, F., Hsu, S.Y., Wang, S.C., 2004. Purifications of

multi-walled carbon nanotubes by microwave digestion method. Diamond Relat. Mater. 13, 1182-1186.

da Rocha, A.M., Salomão de Freitas, D.P., Burns, M., Vieira, J.P., de la Torre, F.R., Monserrat, J.M., 2009. Seasonal and organ variations in antioxidant capacity detoxifying competence and oxidative damage in freshwater and estuarine fish from Southern Brazil. Comp. Biochem. Physiol. C 150, 512-520.

da Rocha, A.M., Ribas Ferreira, J., Barros, D.M., Brandão, T.C., Bogo, M.R., Oliveira, S., Geraldo, V., Lacerda, R.G., Ferlauto, A.S., Ladeira, L.O, Pinheiro, A.S., Ladeira, L.O., Pinheiro, M.V.B., Monserrat, J.M. 2013. Gene expression and biochemical responses in brain of zebrafish *Danio rerio* exposed to organic nanomaterials: Carbon nanotubes (SWCNT) and fullerenol ($C_{60}(OH)_{18–22}(OK_4)$). Comp. Biochem. Physiol. A 165, 460-467.

Ellman, G.L., Courtney, K.D., Andres, V.Jr., Feather-Stone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88-95.

Hermes-Lima, M., Zenteno-Savin, T., 2002. Animal response to drastic changes in oxygen availability and physiological oxidative stress. Comp. Biochem. Physiol. C 133, 537-566.

Monserrat, J.M., Martínez, P.E., Geracitano, L., Amado, L.L., Martins, C., Martinez, G., Pinho, G., Lopes, L., Chaves, I.S., Ferreira-Cravo, M., Ventura-Lima, J., Bianchini, A., 2007. Pollution biomarkers in estuarine animals: Critical review and new perspectives. Comp. Biochem. Physiol. C 146, 221-234.

Mouchet, F., Landois, P., Puech, P., Pinelli, E., Flahaut, E., Gauthier, L., 2010. Carbon nanotube ecotoxicity in amphibians: Assessment of multiwalled carbon nanotubes and comparison with double- walled carbon nanotubes. Nanomedicine 5, 963-974.

Nilsson, G.E.,1990. Long-term anoxia in crucian carp: Changes in the levels of amino acid and monoamine neurotransmitters in the brain, catecholamines in chromaffin tissue, and liver glycogen. J. Exp. Biol. 150, 295-320.

Kopecka, J., Pempkowiak. J., 2008. Temporal and spatial variations of selected biomarker activities in flounder (*Platichthys flesus*) collected in the Baltic proper. Ecotox. Environ. Safe. 70, 379-391.

Kunzmann, A., Anderson, B., Thurnherr, T., Krug, H., Scheynius, A., Fadeel, B., 2011. Toxicology of engineered nanomaterials: Focus on biocompability, biodistribution and biodegradation. Biochem. Biophys. Acta. 1810, 361-373.

Jorio, A., Pimenta, M.A., Filho, A.G.S., Saito, R., Dresselhaus, G., Dresselhaus, M.S., 2003. Characterizing carbon nanotube samples with resonance Raman scattering. N. J. Phys. 5, 139.1– 139.17.

Liu, Y., Zhao, Y., Sun, B., Chen, C., 2012. Understanding the toxicity of carbon nanotubes. Acc. Chem. Res. 46, 702-713.

Manke, A., Wang, L., Rojanasakul, Y., 2013. Mechanisms of nanoparticle induced-oxidative stress and toxicity. Biomed Res. Int. 2013, 1-15.

Nakanishi, H., Takeda, H. 1973. The possible role of adenosine triphosphate in chemical transmission between the hypogastric nerve terminal and seminal vesicle in the guinea-pig. The Japanese Journal of Pharmacology 23: 479-490.

Oakes, K.D., Van der Kraak, G.J., 2003. Utility of TBARS assay in detecting oxidative stress sucker (*Catostomus commersoni*) populations exposed to pulp mill effluent. Aquat. Toxicol. 63, 447-463.

Parks, A.N., Portiz, L.M., Schiers, A., Washburn, K.M., Perron, M.M., Burgess, R.M., Ho, K.T., Chandles, T., Ferguson, L., 2013. Bioaccumulation and toxicity of single-walled carbon nanotubes to benthic organisms at the base of the marine food chain. Environ. Toxicol. Chem.32, 1270-1277.

Powers, C.M., Levin, E.D., Seidler, F.J., Slotkin, T.A., 2011. Silver exposure in developing zebrafish produces persistent synaptic and behavioral changes. Neurotox. Teratol. 33, 329-332.

Senger, M.R., Rico, E.P., Arizi, M.B., Rosemberg, D.B., Dias, R.D., Bogo, M.R., Bonan, C.D., 2005. Carbofuran and malathion inhibit nucleotide hydrolysis in zebrafish (*Danio rerio*) brain membranes. Toxicology 112, 107-115.

Shvedova, A.A., Pietroiust, A., Fadeel, B., Kagan, V.E., 2012. Mechanisms of carbon nanotube-induced toxicity: Focus on oxidative stress. Toxicol. Appl. Pharmacol. 261, 121-133.

Smith, C.J., Shaw, B.J., Handy, R.D., 2007. Toxicity of single walled carbon nanotubes to rainbow trout (*Oncorhynchus mykiss*): Respiratoty toxicity, organ pathologies and other physiological effects. Aquat. Toxicol. 82, 94-109.

Tabet, L., Bussy, C., Amara, N., Setyan, A., Grodet, A., Rossi, M.J., Pairon, J.C., Boczkowski, L., Lanone, S., 2009. Adverse effects of industrial multiwalled carbon nanotubes on human pulmonary cells. J. Environ. Health. 72, 60-73.

Takahashi, Y., Mizuo, K., Shinkai, Y., Oshio, S., Takeda, K., 2010. Prenatal exposure to titanium dioxide nanoparticles increases dopamine levels in the prefrontal cortex and neostriatum of mice. J. Toxicol. Sci. 5, 749-756.

Wang, J., Rahman, M.F., Duhart, H.M., Newport, G.D., Patterson, T.A., Murdock, R.C., Hussain, S.M., Schlager, J.J., Ali, S.F., 2009. Expression changes of dopaminergic system-related genes in PC12 cells induced by manganese, silver, or copper nanoparticles. NeuroToxicology 30, 926-933.

Wang, Z., Zhao, J., Li, F., Gao, D., Xing, B., 2009. Adsortion and inhibition of acetylcholinesterase by different nanoparticles. Chemosphere 77: 67-73.

White, C.C., Virnes, H., Krejsa, C.M., Botta, D., Kavanagh, T.J. 2003. Fluorescence-based microtiter plate assay for glutamate-cysteine ligase activity. Anal. Biochem. 318, 175-180.

Wohlfart, S., Gelperina, S., Kreuter, J., 2012. Transport of drugs across the blood-brain barrier by nanoparticles. J. Cont. Rel. 161, 264-273.

Worek, F., Reiter, E.G., Eyer, E.P., Szinics, E.L., 2002. Reactivation kinetics of acetylcholinesterase from different species inhibited by highly toxic organophosphates. Arch. Toxicol. 76, 523-529.

Wu, J., Wang, C., Sun, J., Xue, Y., 2011. Neurotoxicity of silica nanoparticles: Brain localization and dopaminergic neurons damage pathways. ACS Nano 6, 4476-4489.

Yang, S,T., Guo, W., Deng, X.Y., Wang, H,F., Sun, H.F., Liu, Y.F., Wang, X., Wang, W., Chen, M., Huang, Y.P., Sun, Y.P., 2007. Biodestribution of pristine single-walled carbon nanotubes *in vivo*. J. Phys. Chem. 111, 17761-17764.

Zar, J.H., 1984. Biostatistical Analysis. Prentice Hall, New Jersey,pp. 130-155.

Zhang, L., Bai, R., Liu, Y., Meng, L., Li, B., Wang, L., Xu, L., Le Guyader, L., Chen, C., 2012. The dose-dependent toxicological effects and potential perturbation on the neurotransmitter secretion in brain following intranasal instillation of copper nanoparticles. Nanotoxicoloy 6, 562-575.

Zhao, X., Liu, R., 2012. Recent progress and perspectives on the toxicity of carbon nanotubes at organism, organ, cell, and biomacromolecule levels. Environ. Int. 40, 244-256.

Zimmermann, H., 2011. Ectonucleotidases: Some recent developments and note on nomenclature. Drug Develop. Res. 52: 44-56.

Figure legends

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. 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) Asterisk indicates statistical differences between indicated treatments ($p \leq 0.05$). To ACAP a relative area was calculated dividing area difference (with and without ABAP) by area without ABAP (background area). Larger area means lower antioxidant capacity.

Fig. 3. Dopamine (DOPA) (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) Asterisk indicates statistical differences between indicated treatments ($p \leq 0.05$).

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) Asterisk indicates statistical differences between indicated treatments ($p \leq 0.05$).

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) Asterisk indicates statistical differences between indicated treatments ($p \leq 0.05$).

Figure 1

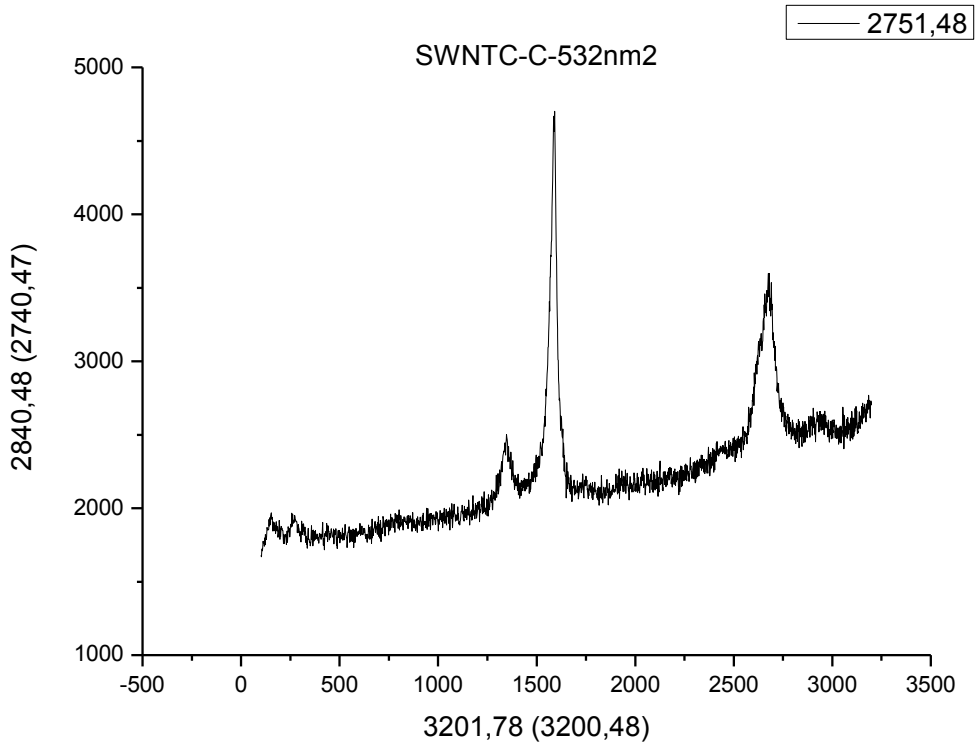

2751,48
SWNTC-C-532nm2
2840,48 (2740,47)
3201,78 (3200,48)
5000
4000
3000
2000
1000
-500
0
500
1000
1500
2000
2500
3000
3500

Figure 2

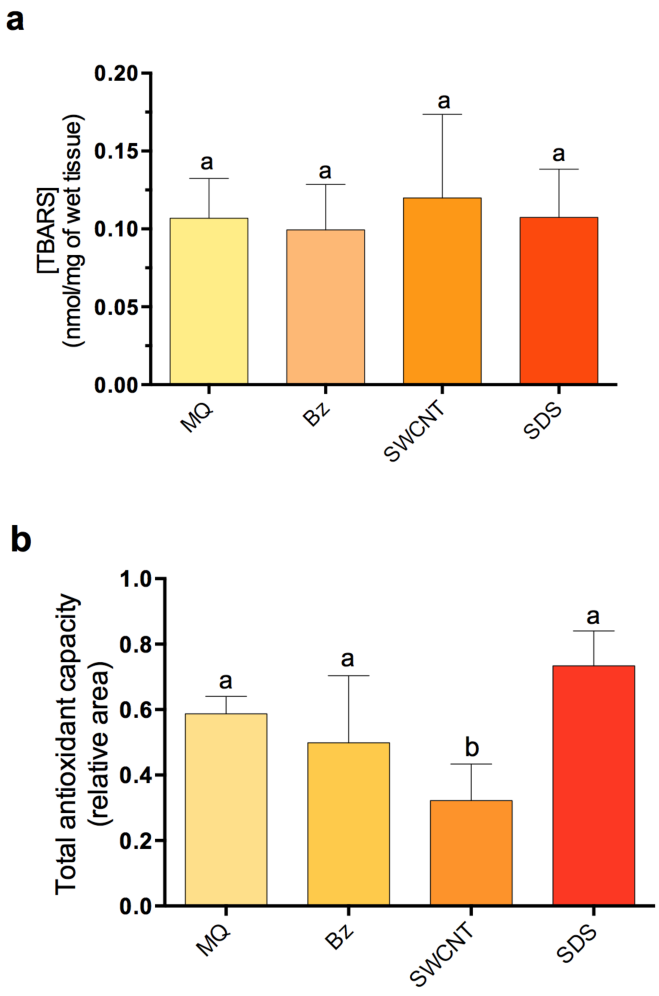
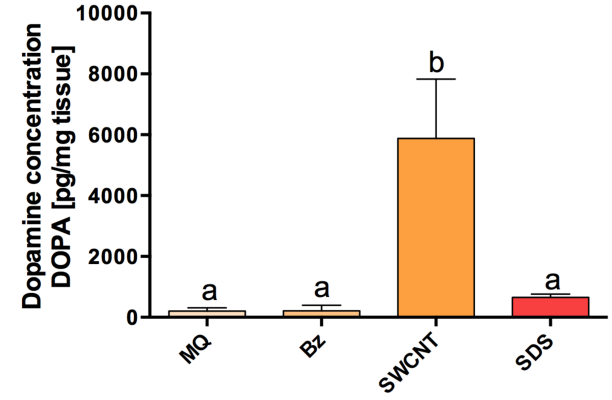

a
[TBARS]
(nmol/mg of wet tissue)
0.20
0.15
0.10
0.05
0.00
a
a
a
a
MQ
Bz
SWCNT
SDS
b
Total antioxidant capacity
(relative area)
1.0
0.8
0.6
0.4
0.2
0.0
a
a
b
a
MQ
Bz
SWCNT
SDS

Figure 3


Dopamine concentration
DOPA [pg/mg tissue]
10000
8000
6000
4000
2000
0
a
a
b
a
MQ
Bz
SWCNT
SDS

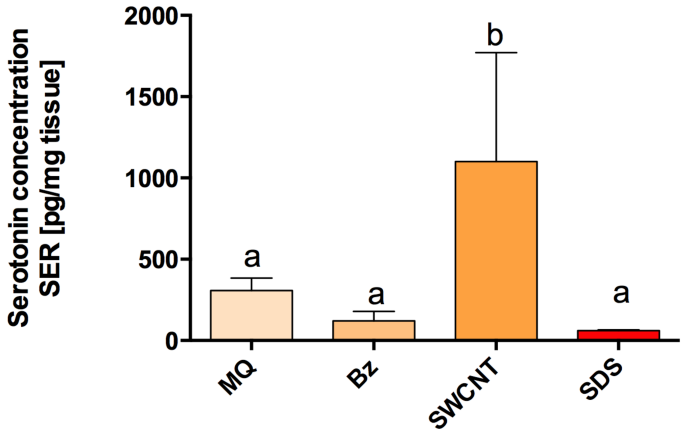

Serotonin concentration
SER [pg/mg tissue]
2000
1500
1000
500
0
a
a
b
a
MQ
Bz
SWCNT
SDS

Figure 4

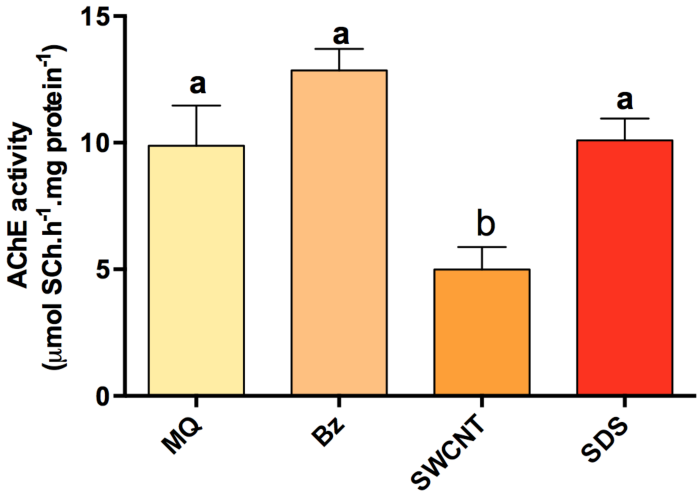
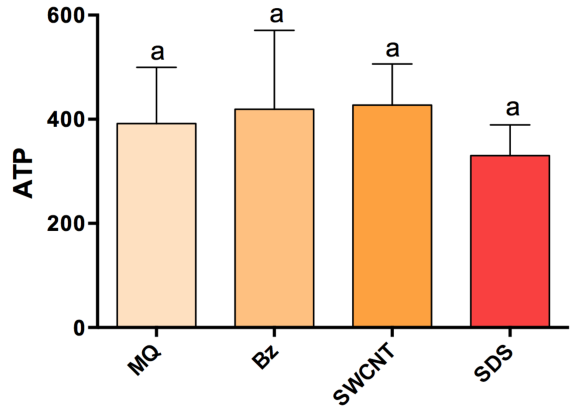
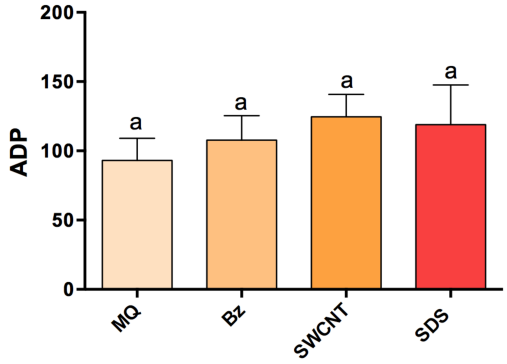
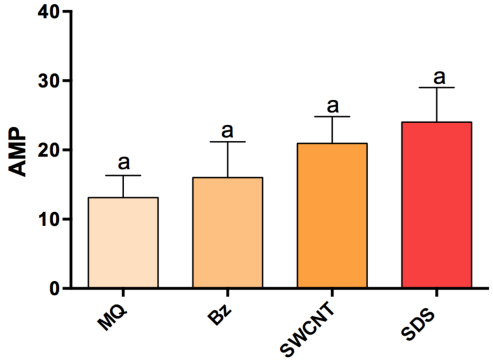

AChE activity
(µmol SCh.h-1.mg protein-1)
15
10
5
0
a
a
b
a
MQ
Bz
SWCNT
SDS

Figure 5


ATP
600
400
200
0
a
a
a
a
MQ
Bz
SWCNT
SDS


ADP
200
150
100
50
0
a
a
a
a
MQ
Bz
SWCNT
SDS


AMP
40
30
20
10
0
a
a
a
a
MQ
Bz
SWCNT
SDS

8. Artigo 3

Responses of antioxidant and reference genes in zebrafish *Danio rerio* embryos exposed to single walled carbon nanotubes

(a ser submetido à revista Nanotoxicology)

RESPONSES OF ANTIOXIDANT AND REFERENCE GENES IN ZEBRAFISH *Danio rerio* EMBRYOS EXPOSED TO SINGLE WALLED CARBON NANOTUBES

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Abstract

One of the most discussed mechanisms, in addition to the health effects caused by environmental particles, is the ability of nanomaterials to promote oxidative stress. The increased used of carbon nanotubes (CNT) in the industry, makes essential the need of *in vivo* toxicological studies with these nanomaterials in order to evaluate their potential toxicity. Thus, we explored the role of antioxidant genes on single walled carbon nanotubes (SWCNT) toxicity in zebrafish. After treating the zebrafish embryos with a SWCNT suspension, it was evaluated the expression of the catalytic subunit of glutamate cysteine ligase (GCLc) and glutathione peroxidase 1 (GPx1) in relation to four reference genes: β-actin, eukaryotic translation initiation factor 1b (eif1b), mitochondrial ATP synthase subunit b (atp5f1) and tyrosine 3-monooxygenase/tryptophan 5-mooxygenase activation protein theta polypeptide b (ywhaqb). RT-qPCR analysis demonstrated that β–actin gene was not a stable reference gene, since the expression of this gene when individually analyzed showed a significant increments in its own gene expression as compared to other treatments (1, 10 and 50 ppm of SWCNT suspension). The expression of GCLc and GPx1 antioxidant genes were significantly increased in embryos treated with 10 and 50 ppm of SWCNT as compared to control and the lowest concentration of 1 ppm of SWCNT suspension. Given these results, we can assert that the development of zebrafish embryos is affected by up-regulation of genes related to oxidative stress and by deregulation expression of β –actin, an essential gene required for embryonic development.

Key words: nanotoxicology, gene expression, nanomaterials, carbon nanotubes, zebrafish, antioxidant genes

1. Introduction

The potential environmental impact of single wall (SWCNT) and multi wall carbon nanotubes (MWCNT) nanotubes is highly relevant because the various applications of these nanomaterials has promoted an increase in its production and this event may augment the release in emissions of these nanomaterials to the air, soil and sediments (Parks et al., 2013), which can reach the groundwater, water reservoirs and river systems. Besides, the nanotubes are one of the least biodegradable materials being totally insoluble in water when in it pristine form and lipophilic in nature (Wu et al., 2006). Hence, they can to accumulate in the food chain through its capture by microbial communities and roots, and thus being retained in plants tissues (Oberdörster et al, 2006) or being toxic to human cells, another animals and bacteria (Jaisi et al., 2008).

The oxidative stress is a common pathway of toxicity and diseases and an organism may be subject to it through multiple mechanisms, induced by oxidant agents as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxil radicals ($HO^{\cdot}$), between others. These reactive oxygen species (ROS) are produced endogenously or by several foreign agents, including nanotubes that among other responses can inhibit the production of antioxidant molecules as reduced glutathione (GSH) (Zhao et al., 2012). GSH is responsible to maintain the redox balance, acting on metabolites detoxification and on ROS, which are associated with chemical expositions and diseases (Usenko et al., 2008). The synthesis of GSH from its constituent amino acids involves the actions of two ATP-dependent enzymes, γ-glutamylcysteine ligase (GCL) and GSH synthetase. GCL, the rate-controlling

enzyme in the overall pathway, is a heterodimer composed of a catalytic (GCLc; 73 kDa) and a modulatory (GCLm; 30 kDa) subunit (Suh et al., 2004).

A first and rapid effect of nanotubes on cells is the generation of ROS, inducing oxidative stress, which is seen as a key factor in cellular functionality (Shvedova et al., 2012). On the other hand, since the cellular and nuclear membranes can be affected by nanotubes, the transcription factor nuclear factor-erytroid 2 p45-related factor 2 (Nrf2), which active antioxidant genes, can also be changed. This situation was reported in a study where intraperitoneal injections of SWCNT were applied in adult zebrafish and it was possible to observe an increase on brain Nrf2 expression compared to control, showing us that nanotubes have a pro-oxidant behavior (Da Rocha et al., 2013). One particular area of concern for nanomaterials' uses is the vulnerability of embryos of fishes to oxidative stress as observed by Usenko et al. (2008) that examined the effect of fullerene (C_{60}) on zebrafish embryos, showing several genes, known to be involved in an oxidant stress response, being up-regulated after exposure to C_{60}., including glutathione-S-transferase pi (GST-pi) and GCLc, both directly related to glutathione metabolism (Handy et al., 2011). Contrary to these findings, the mRNA levels of glutathione peroxidase 1 (GPX1) in zebrafish liver tissues was reduced by silver nanoparticles (AgNPs) at 120 mg Ag/L compared to controls (Choi et al., 2010), a responses that can affect H_2O_2 concentration, since GPX is one of the enzymes required for its degradation.

Some studies show concern about nanotubes toxicity promoted by different types of nanotubes, such as the high inhibition of cellular proliferation and serious morphologic damages on developing embryo caused by MWCNT

acute toxicity (Chen et al. 2012) and a decrease total antioxidant capacity in brain from zebrafish treated with intraperitoneal injection of SWCNT (Da Rocha et al., 2013). In this study, we sought to determine the antioxidant genes expression profile in zebrafish to mediate the toxicity caused by SWCNT exposure.

2. Materials and Methods

2.1. Fish care

Adult zebrafish *Danio rerio* were maintained in a recirculating AHAB system (Aquatic Habitats, Apopka, FL, USA) at 28 °C under a 14:10 light: dark cycle. Adult fish were fed brine shrimp and a mix of Cyclop- eeze (Argent Chemical Laboratories, WA, USA) and Zeigler's Adult Zebrafish Complete Diet (Aquatic Habitats).

Embryos were collected after natural spawning of adult zebrafish and were maintained in 30% Danieau's solution (Nasevicius and Ekker, 2000) in an incubator under the same temperature and photoperiod conditions of adults.

2.2. Characterization of SWCNT

The nanotubes were purchased from Cheap Tubes Inc. To characterize the morphology and structure of the SWCNT, Raman Spectroscopy and Transmission Electron Microscopy (TEM) were used. For these analyze, equipment Raman spectroscopy, Renishaw Model inVia Spectrometer was used. The experiments were performed at room temperature in the range 0-

3100 cm^{-1}, using a laser of 532 nm wavelength (Jorio et al., 2003). TEM used was JEOL model JEM-1200ExII.

2.3. Dispersing single wall carbon nanotubes in gum Arabic

Suspension of SWCNT was prepared with gum arabic (GA), a complex mixture of saccharides and glycoproteins obtained from the acacia tree. Once there was no cytotoxic effect of SWCNT suspensions on either the prokaryotic, bacterial (*Escherichia coli*), or eukaryotic cell types in a study that assayed SWCNT dispersed using GA (Alpatova et al., 2010) , we selected GA as the dispersant to carry out our study with embryos treated with nanotubes.

The aqueous solution of GA was prepared in 50 mL of water and the pH of the mixture adjusted to 7.0 with 0.1 M HCl. This solution was left to settle for 24 h and then 40 mL of supernatant was collected for use in toxicity study. SWCNT were added to the above solutions to result in a 1 mg (SWCNT)/mL suspension and then sonicated in water bath for 20 min. After the sonication, SWCNT suspensions were divided into 12 mL aliquots and each aliquot was transferred into 15 mL centrifuge tube. After 24 h standing time, the top 4 mL of each suspension was used in this study (Alpatova et al., 2010).

2.4. SWCNT exposure

At 24 hours post fertilization (hpf), healthy embryos were manually dechorionated (Usenko et al., 2008; Van Tiem and Di Giulio, 2011) and dosed in replicate pools of ten embryos, with three replicates per treatment, in 20 mL

glass vials containing 5 ml 30% Danieau's solution with SWCNT. Selected concentrations were 1, 10 and 50 ppm of nanotubes, and as a control, embryos without nanotubes, just immerses in Danieau's solution, were used. Dosed embryos were maintained in an incubator at 28°C until the time of deformity assessment or the time to RNA extraction. Those concentrations were selected on the basis of a previous study that evaluated toxicological effects of multi walled carbon nanotubes (MWCNT) on zebrafish embryos (Chen et al., 2012).

2.5. Dosing for deformities

At 24 hpf, hatched embryos were treated as described in section 2.3. At 96 hpf were removed from the dosing solution, rinsed with with 30% Danieau's solution and anesthetized with MS-222. Fish were placed on depression slides in the left lateral position in 3% methylcellulose and were imaged using light microscopy under 50 x (Zeiss Axioskop, Thornwood, NY, USA).

2.6. Total RNA extraction and reverse transcription

After 24 hours, no death or deformities were observed at this time point when embryos were rinsed with 30% Danieau's solution, and then the same embryos were transferred to eppendorfs without Danieau' solution or SWCNT. Those eppendorfs immediately were placed in liquid nitrogen. After that, the samples were thawed on ice and homogenized with a sterile hand-held homogenizer for 30 s, and RNA was extracted according to the RNA-Bee

protocol (Tel-Test Inc., Friendswood, TX, USA). RNA quantity was analyzed spectrophotometrically using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE). The Omniscript complementary DNA (cDNA) synthesis kit for Reverse Transcription (Qiagen) was used according to the manufacturer's instructions using 500 ng of RNA, random hexamers, and RNAse inhibitor and carried out in a thermocycler for 1 h at 37°C. Resulting cDNA was diluted to a working concentration of 2 ng/l.

2.7. RT-qPCR

The expression of the following genes was examined: catalytic subunit of glutamate cysteine ligase (GCLc) and glutathione peroxidase 1 (GPx1) and to test the accuracy of qPCR results, the interest genes were normalized with different reference genes: β -actin, eukaryotic translation initiation factor 1b (eif1b), mitochondrial ATP synthase subunit b (atp5f1) and tyrosine 3-monooxygenase/tryptophan 5-mooxygenase activation protein theta polypeptide b (ywhaqb). GCLc and GPX1 primers were published previously (Van Tiem and Di Giulio, 2011; Garner and Di Giulio, 2012). The choice of reference genes was based on a doctoral thesis (unpublished data), which showed these genes as the most stable when zebrafish embryos were treated with polychlorinated biphenyls (PCB).

Each 25-µL RT-hqPCR reaction consisted of 12.5 µL SYBR Green PCR Master Mix (Applied Biosystems), 9.5 µL dH2O, 200 nM each forward and reverse primer, and 4 ng cDNA template. The reactions were carried out using an Applied Biosystems 7300 Real-Time PCR System with a thermal

profile of 10 min at 95 °C and 40 replicates of 15 s at 95 °C, 1 min at 60 °C. A dissociation curve was calculated for each sample at the end of each profile to confirm formation of a single product during the reaction. All samples were run in duplicate, and technical replicates were averaged prior to analysis. The ABI PRISM 7300 Sequence Detection System Software, Version 1.1 (Applied Biosystems) was used to carry out data analysis. The average mRNA fold induction of each target gene was calculated by comparing the CT (threshold cycle) of the target gene to that of housekeeking genes according to Livak and Schmittgen (2001).

2.6. Statistical analysis

Values of all measurements were expressed as mean ± 1 standard error. Statistical analysis was performed through analysis of variance (ANOVA) followed by Newman–Keuls test. 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).

3. Results

Four reference genes were selected for analysis from commonly used reference genes and two interest genes were analyzed. Gene names, abbreviations, GenBank accession numbers and primer sequences are listed in Table 1.

The presence of SWCNT characteristic bands observed in Raman spectrum is shown in Fig.1a. Raman spectra of SWCNT showing a distinct peak at about 1.600 cm^{-1} (G-band) corresponding to the graphitic stretch of SWCNT. The images obtained by TEM (Fig.1b) showed the presence of SWCNT.

At 96 hpf, 1, 10 and 50 ppm SWCNT suspensions in GA did not cause any statistically significant deformities as compared to control embryos (Fig. 2a). However, many embryos treated with 10 or 50 ppm SWCNT suspensions kept part of the chorion attached to their body even after hatching, besides to present an irregular swim (Fig. 2b).

qPCR analysis was used to quantify mRNA levels of β-actin, eif1b, atp5f1 and ywhaqb, which were used as reference genes and GCLc and GPx1 were used as interest genes. When these genes were observed in relation to β-actin reference gene, GCLc and GPX1 expression were induced by 10 and 50 ppm SWCNT treatments. Exposure to 10 and 50 ppm induced GCLc expression about 30 and 40 fold over control levels ($p<0.05$), respectively and GPx1 expression about 40 and 50 fold over control levels ($p<0.05$), respectively (Fig. 3a). When these genes were observed in relation to eif1b reference gene, GCLc and GPX1 expression were also induced by 10 and 50 ppm SWCNT treatments. Exposure to 10 and 50 ppm induced GCLc expression about 60 and 50 fold over control levels ($p<0.05$), respectively and GPx1 expression about 75 and 70 fold over control levels ($p<0.05$), respectively (Fig. 3b). In the same way, when those genes were observed in relation to atp5f1 reference gene, GCLc and GPX1 expression were induced by 10 and 50 ppm SWCNT treatments. Exposure to 10 and 50 ppm induced

GCLc expression about 15 and 20 fold over control levels ($p<0.05$), respectively and GPx1 expression about 15 and 25 fold over control levels ($p<0.05$), respectively (Fig. 3c) and, following the same pattern, when those genes were observed in relation to ywhaqb reference gene, GCLc and GPX1 expression were induced by 10 and 50 ppm SWCNT treatments. Exposure to 10 and 50 ppm induced GCLc expression about 20 and 25 fold over control levels ($p<0.05$), respectively and GPx1 expression about 20 and 30 fold over control levels ($p<0.05$), respectively (Fig. 3d).

However, these results seems to be artifacts, since using the equation: 2^{-CT} for all genes, it was observed higher expression for the β-actin gene in the control and 1 ppm SWCNT groups (about 6 and 3.5 fold) over 10 and 50 ppm SWCNT groups ($p<0.05$). When we analyzed the GCLc and GPx1 gene expression, we did not observe any significant difference ($p>0.05$) in the concentrations of SWCNT, which the embryos were exposed as compared to control (Fig. 4a). When eif1b gene was analyzed individually, the control group showed an increased eif1b expression about 1.5 folds over 1 ppm SWCNT group ($p<0.05$) and an increased about 10 fold over 10 and 50 ppm SWCNT ($p<0.05$). For the GCLc gene expression, any significant difference was observed between controls and treatments ($p>0.05$), whereas for GPx1 expression, the control group presented a decreased expression about 3 folds over 10 and 50 ppm SWCNT treatments ($p<0.05$) (Fig. 4b). When atp5f1 gene was analyzed individually, the control group showed any significant difference between the other treatments ($p>0.05$). On the other hand, for GCLc gene expression, the 10 ppm SWCNT group showed an expression increased in relation with other treatments ($p<0.05$) and for GPx1 expression, exposure to

10 and to 50 ppm SWCNT had an expression increased about 5 and 4 fold over control and 1 ppm SWCNT group ($p<0.05$), respectively (Fig. 4c). Finally, when ywhaqb gene was analyzed individually, the control group presented an increase expression about 5 folds over 10 and 50 ppm SWCNT treatments ($p<0.05$). A different situation is observed for GCLc expression: the control had the expression decreased about 4 fold compared to 10 and 50 ppm SWCNT groups ($p<0.05$), and for GPx1 expression, exposure to 10 ppm and to 50 ppm SWCNT had an increased expression about 5 and 4 fold over control and 1ppm SWCNT treatments ($p<0.05$), respectively (Fig. 4d).

4. Discussion

Zebrafish is a commonly used model organism in developmental toxicity studies, including gene expression studies, particularly qPCR. There are many papers using qPCR in zebrafish that show the β-actin as one of the most popular reference gene (Jonge et al, 2007; Bunnell et al, 2011). However, based on our study, the β-actin gene is not a stable reference gene as its expression was affected by SWCNT treatment.

Several reference genes have been historically used for gene expression analysis (Bustin, 2000; Tang et al., 2007). Reference gene selection is critical to accurate assessment of changes in gene expression. However, experimental evidence shows that many common reference genes do not exhibit constant expression under all experimental conditions and the use of inappropriate reference genes can result in highly misleading false-negative or false-positive results, obscuring true biological effects (McCurley and

Callard, 2008). The use of a single reference gene has been shown to lead to incorrect normalization calculations of up to 3- and 6-fold in expression studies in various human tissues (Vandesompele et al., 2002). Thus, the use of only one reference gene for normalization of gene expression studies should not be considered sufficient. Thus, it was tested the accuracy of qPCR results after normalization with different reference genes, being observed an exacerbated up-regulalation of the target GCLc and GPx1 genes when embryos were treated with a SWCNT suspension. These responses led us to question whether we were not faced with false-positive results. When the expression levels were normalized to reference genes, GCLC and GPx1 expression was up-regulated about 20 times or more. The analysis of CT for each gene (targets and reference) showed that the β-actin gene had an increased expression in the control group. As far as that β-actin gene alone had this increased expression, promoted by SWCNT, if it is used as a reference gene, the expression of the gene of interest has a tendency to increase.

β-actin specifically controls cell growth and migration (Bunnell et al., 2011) and it is known that its overexpression alters cell morphology and motility (Peckham et al., 2001). At 96 hpf, when we analyze the possible deformations in embryos caused by SWCNT suspension, we observed that despite not having any evidence of deformation in zebrafish, many embryos have irregular swimming and remnants from their own chorion attached to the body, even after the hatching. This behavior may be due the β-actin, which can promote a deregulated development.

β-actin plays an important role in development, and that also has been observed in a study with β-actin-knockout mice which were early embryonic lethal, indicating that β-actin is an essential gene required for embryonic development (Bunnell et al., 2011).

Eif1b gene also does not seem to be a good reference gene as well as β-actin, once the control it has increased its expression in relation to other treatments and on that account was not stable enough to be used as a stabilizer gene in this study with SWCNT. Also ywhaqb gene has similar behavior to eif1b since control ywhaqb gene also has its expression increased in relation to the highest SWCNT treatments (10 and 50 ppm) of this same gene.

Given that, atp5f1 gene seems to be the most stable gene for this study with carbon nanotubes. The control atp5f1 gene did not increase in relation to other SWCNT treatments of 1, 10 and 50 ppm of this same gene. When we compare the expression of this gene in relation to interest genes, we could observe an increased expression of GCLc and GPx1 genes in the SWCNT treatments of 10 ppm, and, 10 and 50 ppm, respectively. In the same way, in a study using zebrafish microarray, Usenko et al. (2008), by analyzing two early time points during development, were able to identify impacts that persist over multiple stages of development. These researchers found that many of the genes known to be involved in an oxidative stress response were up-regulated in embryonic zebrafish after exposure to C_{60}. In particular, two genes were significantly up-regulated: GCLc and GSTpi, the latter involved in the phase II metabolism conjugation of GSH to electrophilic xenobiotics.

In conclusion, once the genes of GCLc, GPx1 analyzed as atp5f1 gene, the most stable reference in this study, and since all reference genes had their expression significantly increased when the embryos were treated with SWCNT, it is possible to say that these genes may affect the development of zebrafish embryos, interfering in the normal growth through up-regulation of antioxidant genes or by β-actin up-regulation.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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5. References

Alpatova, A., Shan, W., Babica, P., Upham, B.L., Rogensues, A.R., Masten, S.J., Drown, E., Mohanty, A.K., Alocilja, E.C., Tarabara, V.V. 2010. Single-walled carbon nanotubes dispersed in aqueous media via non-covalent functionalization: Effect of dispersant on the stability, cytotoxicity, and epigenetic toxicity of nanotube suspensions. Water Research 44: 505-520.

Bunnell, T.M., Burbacj, B.J., Shimizu, Y., Ervasti, J.M. 2011. β-Actin specifically controls cell growth, migration, and the G-actin pool. Molecular Biology of the Cell 22: 4047-4058.

Bustion, S.A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. Journal of Molecular Endocrinology 25: 169-193.

Chen, L.Q., Hu, P.P., Zhang, L., Huang, S.Z., Luo, L.F., Huang, C.Z. 2012. Toxicity of graphene oxide and multi-walled carbon nanotubes against human cells and zebrafish. Science China Chemistry 10: 2209-2216.

Choi, J.E., Kim, S., Ahn, J.H., Youn, P., Kang, J.S., Park, K., Y, J., Ryu, D.Y. 2010. Induction of oxidative stress and apoptosis by silver nanoparticles in the liver of adult zebrafish. Aquatic Toxicology 100: 151-159.

Da Rocha, A.M., Ribas Ferreira, J., Marti Barros, D., Pereira, T.C.B., Bogo, M.R., Oliveira, S., Geraldo, V., Lacerda, R.G., Ferlauto, A.S., Ladeira, L.O., Pinheiro, M.V.B., Moserrat, J.M. 2013. Gene expression and biochemical responses in brain of zebrafish *Danio rerio* exposed to organic nanomaterials: carbon nanotubes (SWCNT) and fullerenol ($C60(OH)_{18–22}(OK_4)$). Comparative Biochemistry and Physioloy Part A, 165: 460-467.

Handy, R.D., Bairuty, G.A., Jubory, A.A., Ramsden, C.S., Boyle, D., Shaw, B.J., Henry, T.B. 2011. Effects of manufactured nanomaterials fishes: A target organ and body systems physiology approach. Journal of Fish Biology 79: 821-853.

Jaisi, D.P., Saleh, N.B., Blake, R.E., Elimelech, M. 2008. Transport of single-walled carbon nanotubes in porous media: Filtration mechanisms and reversibility. Environmental Science and Technology 42: 8317-8323.

Jonge, H.J.M., Fehrmann, R.S.N., Bont, E.S.J.M., Hofstra, R.M.W., Gerbens, F., Kamps, W.A., Vries, E.G.E., Van der Zee, A.G.J., Meerman, G.J., Elst, A. 2007. Evidence based selection of housekeeping genes. PlosOne 9: e989.

Jorio, A., Pimenta, M.A., Filho, A.G.S., Saito, R., Dresselhaus, G., Dresselhaus, M.S., 2003. Characterizing carbon nanotube samples with resonance Raman scattering. New Journal of Physics 5: 139.1– 139.17.

Liu, Y., Zhao, Y., Sun, B., Chen, C. 2012. Understanding the toxicity of carbon nanotubes. Accounts of Chemical Research 46: 702-713.

Livak, K.J., Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 25, 402-408.

McCurley, A.T., Callard, G.V. 2008. Characterization of housekeeping genes in zebrafish: Male-female differences and effects of tissue type, developmental stage and chemical treatment. BMC Molecular Biology *9*, 102.

Nasevicius, A., Ekker, S.C. 2000. Effective target gene ‘knockdown’ in zebrafish. Nature Genetics 26: 216-220

Oberdörster, E., Zhu, S., Blickey, T.M., McClellan-Green, P.,Haasch, M.L. 2006. Ecotoxicology of carbon-based engineered nanoparticles: Effects of fullerene (C_{60}) on aquatic organisms. Carbon 44: 1112-1120.

Parks, A.N., Portis, L.M., Schiers, P.A., Washburn, K.M., Perron, M.M. Burgess, R.M., Ho, L.T., Chandler, G.T., Ferguson, P.L. 2013. Bioaccumulation and toxicity of single-walled carbono nanotubes to benthic organisms at the base of the marine food chain. Environmental Toxicology and Chemistry 32: 1270-1277.

Peckham, M., Miller, G., Well, C., Zicha., D., Dunn, G.A. 2001. Specific changes to the mechanism of cell locomotion induced by overexpression of β-Actin. Journal of Cell Science 114: 1367-1377.

Shvedova, A.A., Pietroiusti, A., Fadeel, B., Kagan, V.E. 2012. Mechanisms os carbono nanotube-induced toxicity: Focus on oxidative stress. Toxicology and Applied Pharmacology 261: 121-133.

Suh, J.H., Shenvi, S.V., Dixon, B.M., Liu, H., Jaiswal, A.K., Liu, R.M., Hagen, T.M. 2004. Decline in transcriptional activity of Nrf2 causes age-related loss of glutathione synthesis, which is reversible with lipoic acid. Proceedings of National Academy of Sciences 10: 3381-3386.

Tang, R., Dodd, A., Lai, D., Warren, C., Mcnabb, W.C., Love, D.R. 2007. Validation of zebrafish (*Danio rerio*) reference genes for quantitative real-time RT-PCR normalization. Acta Biochimica et Biophysica Sinica 39: 384-390.

Usenko. C.Y., Stacey, L.,Tanguay, R.L. 2008. Fullerene C_{60} exposure elicits an oxidative stress response in embryonic zebrafish. Toxicology and Applied Pharmacology 229: 44-55.

Wu, Y., Hudson, J., Lu, Q., Moore, J., Mount, A., Rao, A. 2006. Coating walled carbono nanotubes with phospholipids. The Journal of Physical Chemistry 110: 2475-2478.

Van Tiem, L.A., Di Giulio, R.T. 2011. AHR2 knockdown prevents PAH-mediated cardiac toxicity and XRE- and ARE-associated gene induction in zebrafish (*Danio rerio*). Toxicology and Applied Pharmacology 254: 280-287.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3: research0034.

Zar, J.H. 1984. Bioestatistical Analysis. Prentice Hall, New Jersey, pp. 130-155.

Zhao, X., Liu, R. 2012. Recent progress and perspectives on the toxicity of carbon nanotubes at organismo, organ, cell and biomacromolecule levels. Environmental International 40: 244-256.

Figure legends

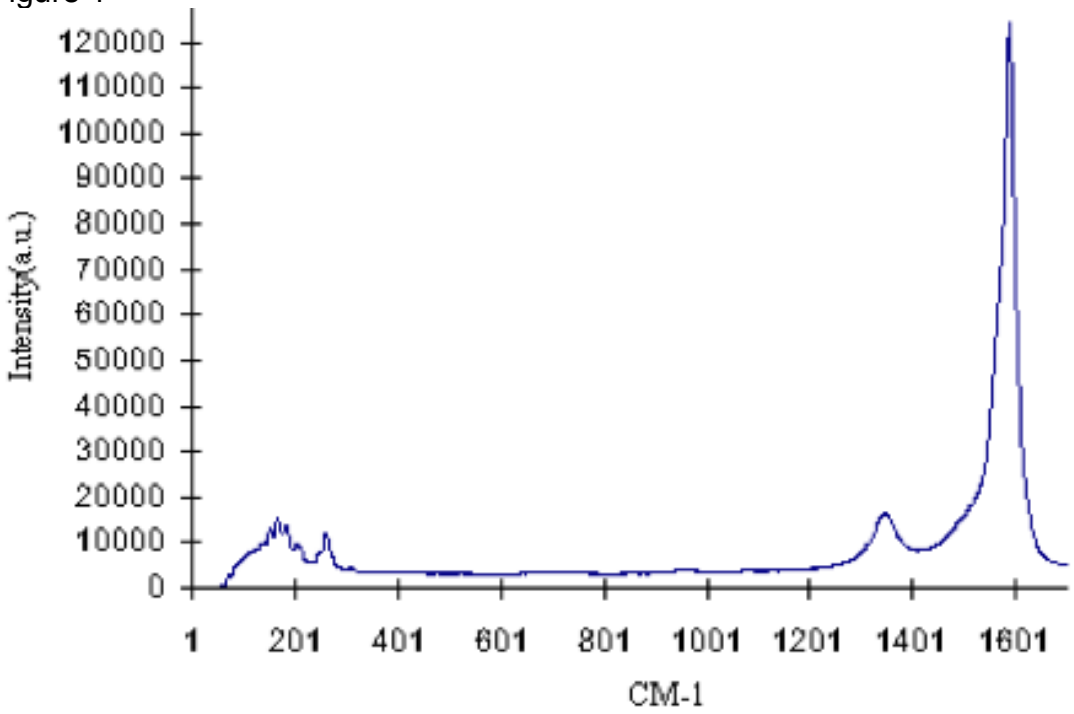
Fig. 1. Raman spectra **(a)** and transmission electronic microscopy (TEM) image **(b)** of single-walled carbon nanotubes (SWCNT). (a) distinct peak at about 1.600 cm^{-1} (G-band), corresponding to the graphitic stretch of SWCNT; (b) presence of fibrous structures, which can be attributed to SWCNT bundles.

Fig. 2. Visual assessment of zebrafish morphology at 96 hpf. **(a)** control zebrafish; **(b)** zebrafish treated with 10 or 50 ppm of SWCNT, presenting part of its chorion attached to the body even after hatching.

Fig. 3. Gene expression of GCLc catalytic subunit and GPx1 when normalized by reference genes: β-actin **(a)**; eif1b **(b)**; atp5f1 **(c)**; ywhaqb **(d)** of zebrafish embryo treated with a SWCNT suspension. Data are expressed as mean +1 standard error (n=3). Identical letters means absence of statistical difference (p≥0,05).

Fig. 4. Gene expression of β-actin, eif1b, atp5f1, ywahqb, GCLc and GPx1 genes expressed as 2^{-CT}. Gene expression of β-actin, GCLc and GPx1 **(a)**; gene expression of eif1b, GCLc and GPx1 **(b)**; gene expression of atp5f, GCLc and GPx1 **(c)**; gene expression of ywhaqb, GCLc and GPx1 **(d)** of zebrafish embryos treated with a SWCNT suspension. Data are expressed as mean +1 standard error (n=3). Identical letters means absence of statistical difference (p≥0,05).

Figure 1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Intensity(a.u.)
1
201
401
601
801
1001
1201
1401
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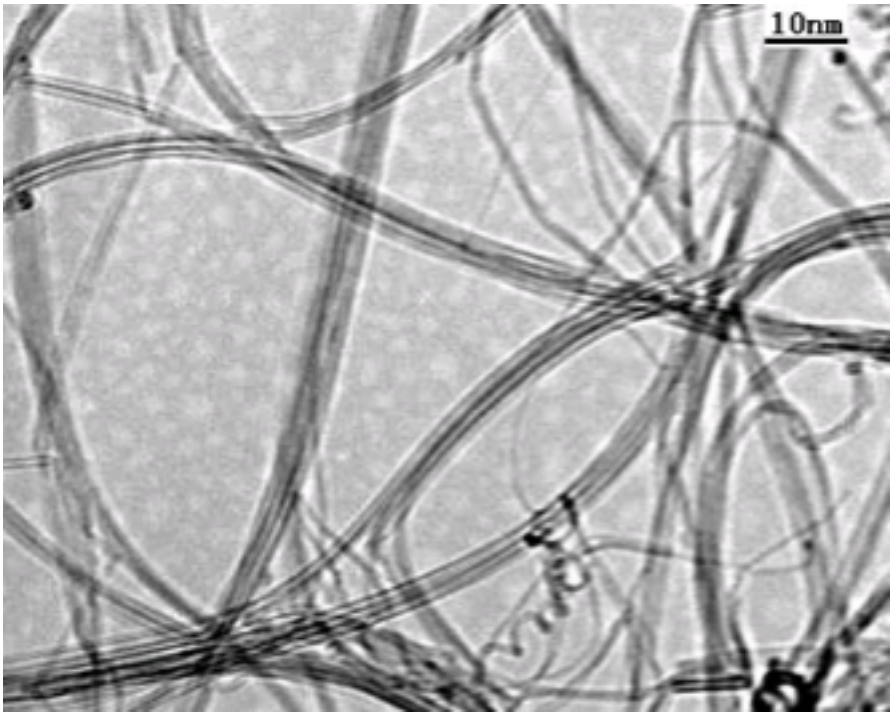
10nm

Figure 2

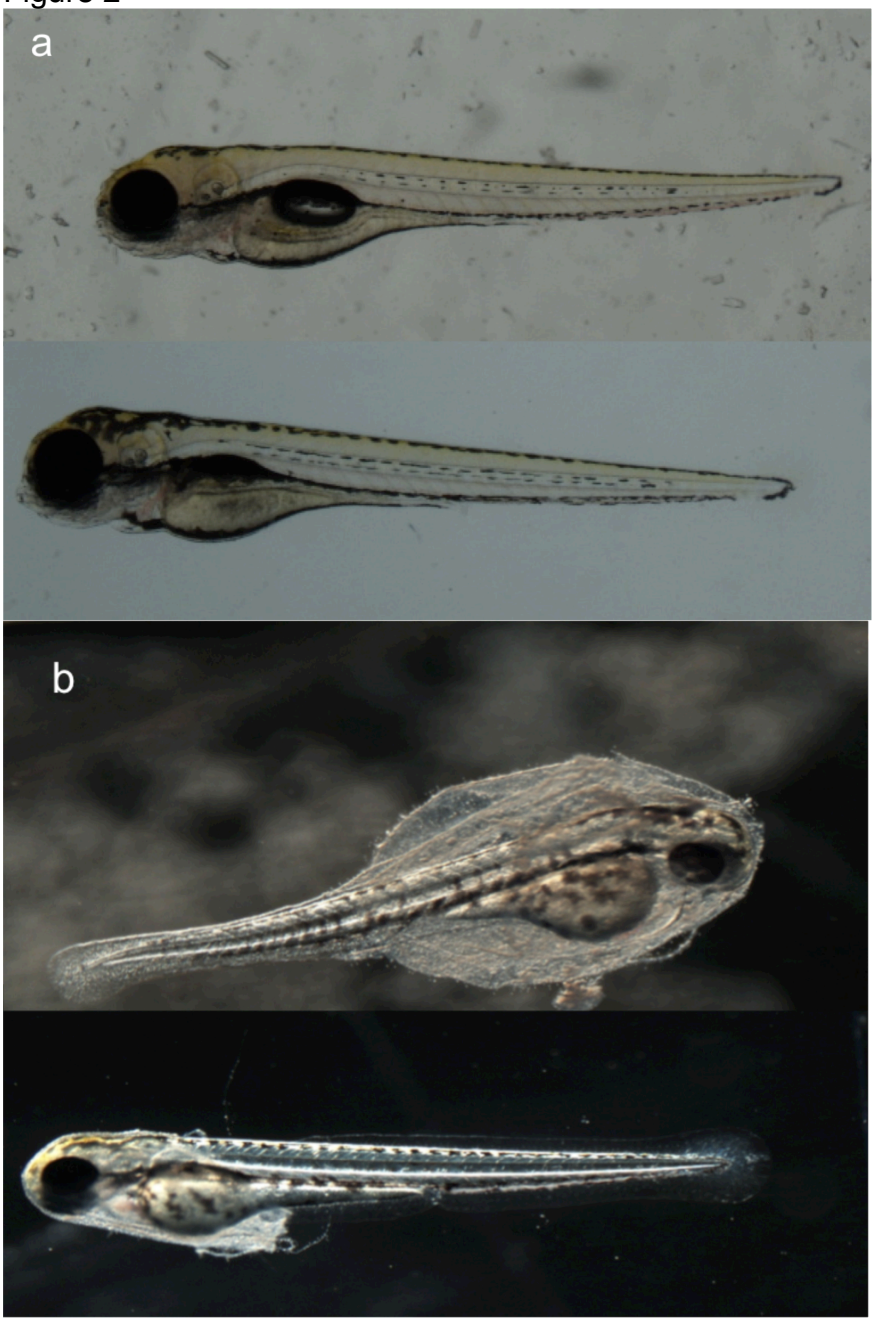

Figure 3

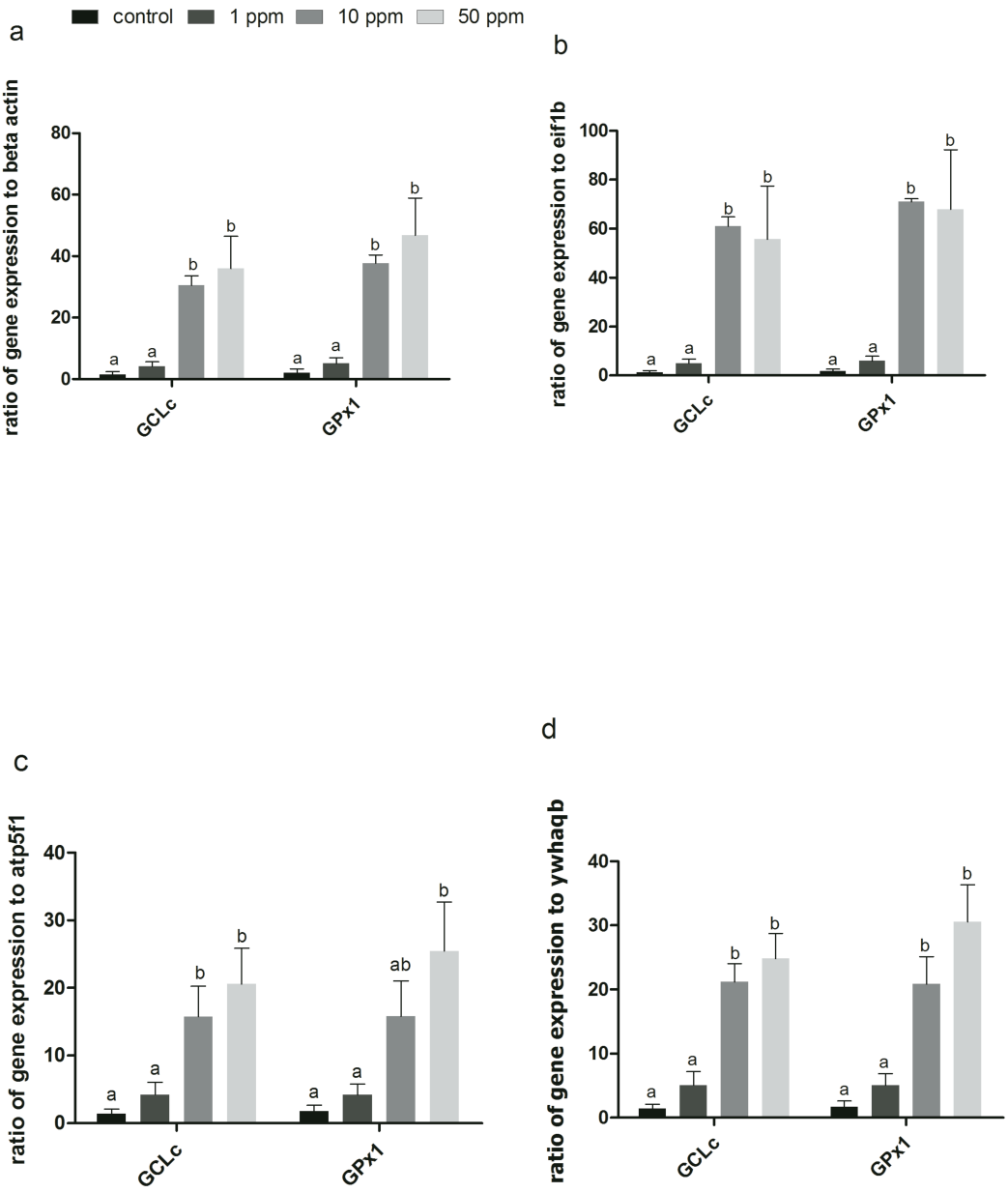
control
1 ppm
10 ppm
50 ppm
a
ratio of gene expression to beta actin
b
ratio of gene expression to eif1b
c
ratio of gene expression to atp5f1
d
ratio of gene expression to ywhaqb
GCLc
GPx1

Figure 4

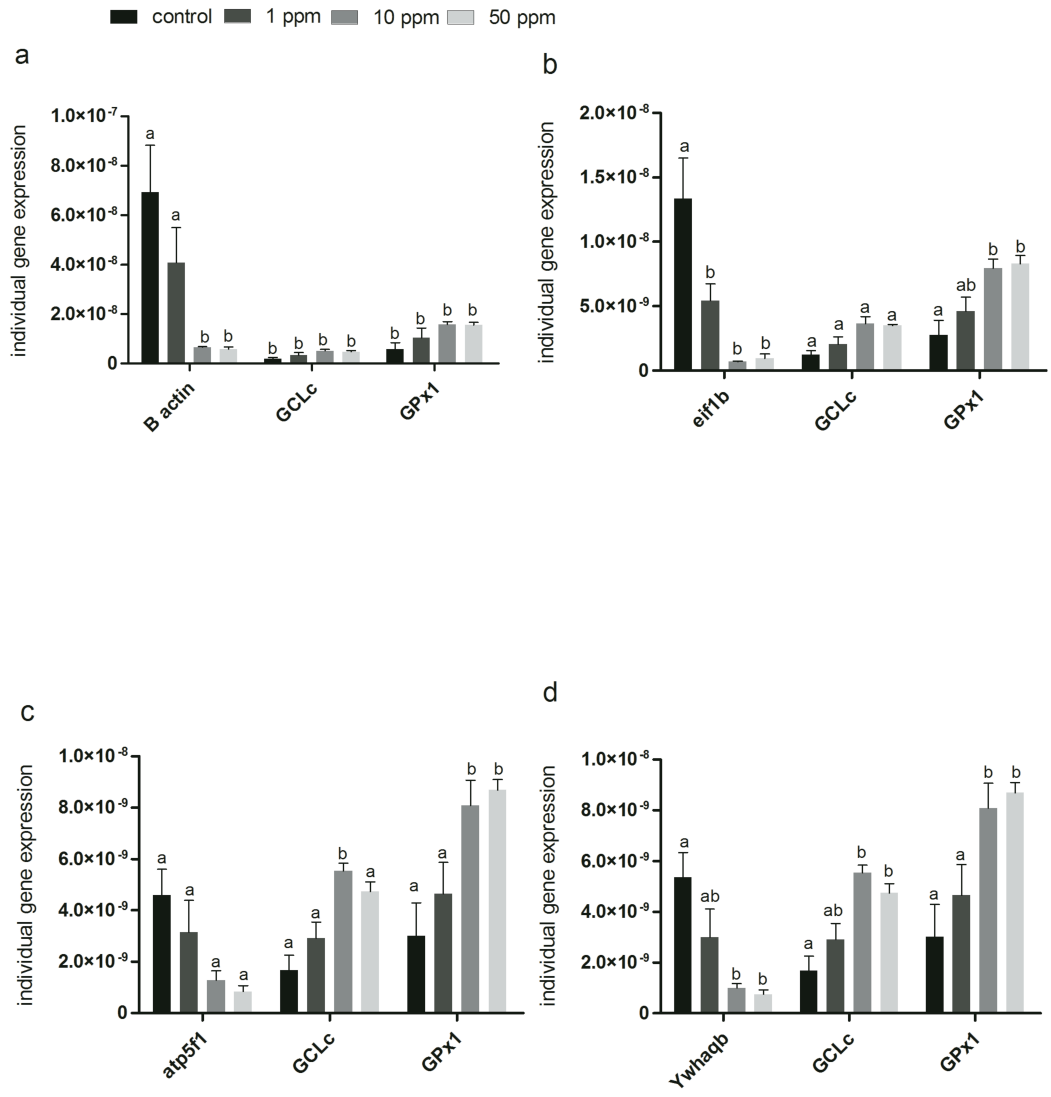
control
1 ppm
10 ppm
50 ppm
a
b
c
d
individual gene expression
B actin
GCLc
GPx1
eif1b
atp5f1
Ywhaqb

Table 1

Primers and GenBank Accession Numbers for qPCR.

Gene	GenBank ID	Forward primer (5'-3")	Reverse primer (5'-3")
β-actin	AF057040	AAGATCAAGATCATTGCTCC	CCAGACTCATCGTACTCCT
eif1b	NM_199588	GCCTTCAAGAAGAAATTTGCC	CCGTGGACTTTGAGCTG
atp5f1	NM_001005960	GAGGTGAAGAAGAGGCTG	CTCCTTCTCCTGCTGTG
ywhaqb	NM_201484	GTCTCGCTCTCAACTTCTC	CAGATGTCCATAATGTGAGGT
GCLc	NM_199277	AAGTGGATGAGGGAGTTTGTTGCC	CTTGTGGAGCAGGTCGTAGTTGAT
GPx1	AW232474	AGATGTCATTCCTGCACACG	AAGGAGAAGCTTCCTCAGCC

9. Considerações Finais

Inclusos nesta tese estão três trabalhos experimentais. No primeiro e segundo trabalhos o peixe adulto *Danio rerio* (Cypridinae) foi utilizado para observar possíveis mudanças na expressão gênica e concentração de neurotransmissores depois dos tratamentos com nanotubos de carbono e fulerol. No terceiro trabalho, embriões de zebrafish foram tratados com suspensão de nanotubos de carbono para observar a expressão de genes antioxidantes.

No **trabalho 1** foi observado que zebrafish adultos, quando tratados com nanotubos de carbono e fulerol tiveram a expressão gênica alterada após tratamento. Depois de 48 horas, o fulerol induziu um aumento na expressão das subunidades catalítica e regulatória da enzima glutamato cisteína ligase quando comparada ao grupo controle, indicando um comportamento antioxidante. A capacidade antioxidante foi menor nos cérebros tratados com fulerol quando comparado ao tratamento com nanotubos.

No **trabalho 2,** o tratamento com nanotubos de carbono aumentou a concentração dos neurotransmissores dopamina e serotonina enquanto a enzima acetilcolinesterase teve sua atividade significantemente reduzida em cérebros de zebrafish expostos a nanotubos de carbono. Estas duas situações evidenciam uma neurotoxicidade induzida por nanotubos de carbono de parede simples.

No **trabalho 3**, a expressão de genes antioxidantes foi significantemente aumentada em embriões tratados com nanotubos de

carbono, além de ser observada um aumento na expressão de genes considerados normalizadores, como β- actina.