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INTERAÇÃO DE NANOTUBOS DE CARBONO DE PAREDES MÚLTIPLAS COM CÁDMIO E SEUS EFEITOS EM CÉLULAS DE PEIXE-ZEBRA IN VITRO

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Tese de doutorado apresentada ao Programa de Pós-graduação em Ecologia e Recursos Naturais da Universidade Federal de São Carlos, como requisito parcial para obtenção do título de Doutor em Ciências.

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RESUMO

A presença de nanotubos de carbono no ambiente aumenta a cada ano, como resultado da sua crescente produção mundial. Em ambientes aquáticos, os nanotubos de carbono podem interagir com outros poluentes com base em suas características de adsorção e alta reatividade química. Metais representam hoje uma das maiores preocupações nos recursos hídricos, dentre os quais o cádmio (Cd) é considerado um dos metais mais prejudiciais para os organismos aquáticos. Este estudo investigou os efeitos dos nanotubos de carbono de paredes múltiplas oxidados (ox-NTCPM) e íons Cd sobre a linhagem celular de fígado de peixe-zebra (ZFL, *zebrafish cell line*) e dois protocolos de interação desses agentes: Protocolo A, ox-NTCPM incubados em meio de cultivo RPMI/L-15 (Roswell Park Memorial Institute e Leibovitz's-15) + 10% soro bovino fetal por 30 min (passo 1) e, subsequente, adição de $CdCl_2$ com incubação por mais 30 min (passo 2); e Protocolo B, ox-NTCPM incubados em solução de $CdCl_2$ por 30 min (passo 1) e subsequente, adição de meio de cultivo RPMI/L-15 + 10% FBS com incubação por mais 30 min (passo 2). Os ox-NTCPM foram caracterizados e sua capacidade de adsorção de Cd foi quantificada, bem como sua estabilidade coloidal em meio de cultivo. A citotoxicidade foi investigada por quatro ensaios e os eventos de necrose e apoptose e fases do ciclo celular foram analisados por citometria de fluxo. Além disso, a internalização celular de Cd nas células ZFL foi quantificada, bem como dez biomarcadores bioquímicos e efeitos genotóxicos nas células. A presença de Cd no sistema não alterou a formação da proteína corona sobre os ox-NTCPM, mas a ordem dos tratamentos (Protocolo A e B) modificou a estabilidade coloidal do complexo ox-NTCPM-Cd e a taxa de adsorção do metal. A presença conjunta de ox-NTCPM e Cd aumentou o conteúdo celular de cádmio em células ZFL e o tratamento com apenas ox-NTCPM promoveu depleção de algumas enzimas antioxidantes e levou a alterações no ciclo celular. Assim, os ox-NTCPM influenciam os efeitos celulares do Cd, devido a um sinergismo de ação, provavelmente relacionado ao efeito característico de nanomateriais denominado "cavalo de Tróia". Os ox-NTCPM elevaram as taxas de apoptose e necrose em células ZFL e ambos protocolos de coexposição com o Cd promoveram aumento nas espécies reativas de oxigênio e efeitos genotóxicos. Dessa forma, foi demonstrado que alterações na ordem de exposição dos ox-NTCPM e Cd em ensaios *in vitro* podem modificar os complexos de NTCPM-metal formados e levar a efeitos biológicos distintos. Adicionalmente, este estudo evidencia o efeito de modulação do ox-NTCPM sobre o Cd e contribui para futuras investigações de coexposição com nanomateriais e poluentes aquáticos e legislações sobre nanomateriais.

Palavras-chave: nanoecotoxicidade, coroa de proteínas, cultura de células, estresse oxidativo e nanossegurança.

ABSTRACT

Carbon nanotubes presence in the environment increases every year as a result of exponential production around the world. In aquatic environments carbon nanotubes can interact with other pollutants based on their nanoadsorbent characteristics. Heavy metals ions represent one of the biggest concerns in water resources nowadays, as a consequence of anthropogenic actions, in which cadmium (Cd) is one of the most harmful metal for aquatic organisms. This study investigates the effects of oxidized multiwalled carbon nanotubes (ox-MWCNT), of Cd ions and two interaction protocols in zebrafish liver cell line (ZFL): Incubation Protocol A, the ox-MWCNT were incubated in RPMI/L-15 (Roswell Park Memorial Institute and Leibovitz's-15) medium + 10% FBS for 30 min (step 1) and, subsequently, the $CdCl_2$ were added and incubated for more 30 min (step 2); and (B) Incubation Protocol B, the ox-MWCNT were incubated with $CdCl_2$ for 30 min (step 1) and, thereafter, the RPMI/L-15 medium + 10% FBS were added and incubated for more 30 min (step 2). Ox-MWCNT were physical and chemical characterized and its Cd adsorption capacity and colloidal stability in cell culture medium was determined. Cytotoxicity was investigated by four assays and the necrosis and apoptosis events and cell cycle were determined using flow cytometer. Cd content and uptake in cells was analyzed and ten biochemical biomarkers and the effects on DNA damage were evaluated. In this study, Cd presence in medium did not interfere in the protein corona composition of ox-MWCNT but the order of interaction of FBS and Cd can change its colloidal stability and metal adsorption rate. This study demonstrate that ox-MWCNT can increase the ZFL content of cadmium. The treatment with only oxidized ox-MWCNT promotes depletion of catalase, glutathione peroxidase and glutathione S-transferase and led to alterations in cell cycle with a reduction of cells in G2/M phase. The ox-MWCNT affects Cd toxicity probably due a “Trojan horse” and synergistic effects of both contaminants and induce apoptosis and necrosis in ZFL cells. We verify that the two co-exposure treatments performed increases the biological cadmium effect in ROS production and led to more genotoxicity damage in DNA. Thus, we show that an alteration in the order protocol method used for ox-MWCNT and Cd interaction in *in vitro* assays can modify the MWCNT-metal complexes formed and led to different biological effects. This study evidence the modulation of ox-MWCNT on Cd biological effects and contributes to future co-exposure investigations and legislations about with nanomaterials interactions with aquatic pollutants.

Keywords: nanoecotoxicity, protein corona, cell culture, oxidative stress and nanosafety.

LISTA DE ABREVIAÇÕES

ANOVA – analysis of variance (inglês)

CAT – catalase

Cd – cádmio

$CdCl_2$ – cloreto de cádmio

CNT - carbon nanotubes (inglês)

DLS – dynamic light scattering (inglês)

FBS – fetal bovine serum (inglês)

GPx – glutathione peroxidase (inglês)

GR – glutathione reductase (inglês)

GSH – reduced glutathione (inglês)

GST – glutathione S-transferase (inglês)

ICP-MS – inductively coupled plasma mass spectrometry (inglês)

ICP-OES – inductively coupled plasma optical emission spectrometry (inglês)

L15 – Leibovitz-15 medium (inglês)

LDH – lactate dehydrogenase (inglês)

MMS – methyl methanesulfonate (inglês)

MTT – (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (inglês)

MWCNT – multiwallet carbon nanotubes (inglês)

NC – negative control (inglês)

NP – nanoparticles (inglês), nanopartículas (português)

NT – nanotubos de carbono

NTCPM – nanotubos de carbono de paredes múltiplas

OTM – Olive tail moment (inglês)

Ox-MWCNT – oxidized multiwallet carbon nanotubes (inglês)

Pex – hidrogen peroxide (inglês)

PI - propidium iodide (inglês)

ROS – reactive oxygen species (inglês), espécies reativas de oxigênio (português)

RPMI – Roswell Park Memorial Institute medium (inglês)

SDS-PAGE – SDS polyacrylamide gel electrophoresis (inglês)

SEM – scanning electron microscope (inglês)

SOD – superoxide dismutase (inglês)

TBARS – thiobarbituric acid reactive substances (inglês)

TEM – transmission electron microscope (inglês)

TGA – thermogravimetric analysis (inglês)

XPS – X-ray photoelectron spectrometer system (inglês)

ZFL –zebrafish cell line from liver (inglês), células de fígado de peixe-zebra (português)

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APRESENTAÇÃO

Este estudo está inserido na área de nanoecotoxicologia com enfoque na interação de nanomateriais com contaminantes aquáticos. Para este trabalho foram escolhidos os nanotubos de carbono de paredes múltiplas e o metal cádmio, e foram averiguados os efeitos de cada agente, bem como os produtos derivados da interação destes potenciais contaminantes ambientais em células de fígado de peixe-zebra pelo sistema de cultivo celular *in vitro*.

O estudo também abrange as áreas de ecologia aplicada, biologia celular, bioquímica, ecotoxicologia e nanotecnologia.

Para melhor organização, esta tese foi dividida em uma introdução geral, seguida por uma revisão bibliográfica e dois artigos científicos originais.

O primeiro estudo foca na caracterização dos nanotubos de carbono oxidados de paredes múltiplas e os efeitos das interações destes com o cádmio em testes de toxicidade comumente utilizados na literatura para sistemas de cultivo *in vitro*. Esse artigo foi publicado na revista Aquatic Toxicology em maio de 2018.

O segundo artigo evidencia a modulação dos nanotubos de carbono oxidados de paredes múltiplas na internalização de cádmio pelas células de peixe-zebra, bem como nos efeitos bioquímicos e genotóxicos do metal e suas interações com o nanomaterial.

Ambos trabalhos são apresentados em inglês e estão expostos nos padrões de publicação, sendo aqui evidenciados como os resultados da tese. Por fim, são descritas as conclusões gerais desta tese e a lista da bibliografia consultada nas seções iniciais.

1 INTRODUÇÃO

Com o crescimento da demanda por novas tecnologias, a produção de nanomateriais tem-se intensificado a cada ano devido às suas características químicas de alta reatividade, possibilitando o uso e aplicações em diversos setores industriais (KAHRU; DUBOURGUIER, 2010). Dentre os nanomateriais, as nanoestruturas de carbono se destacam, uma vez que apresentam um grande potencial de utilização em diversos setores, como a indústria eletrônica e de materiais, e as tecnologias voltadas para a saúde, a agricultura e o meio ambiente (VUKOVIC et al., 2011; MATHUR et al., 2017).

Os nanomateriais de carbono podem apresentar diferentes conformações estruturais, sendo os principais tipos denominados nanotubos de carbono. Esses materiais são formados a partir de uma camada atômica (grafeno) enrolada, com propriedades físicas e químicas que permitem diversas utilizações, como excelentes propriedades de adsorção e alta reatividade química (MARTINEZ et al., 2014). Adicionalmente, por possuírem tamanho em escala nanométrica, esses materiais podem interagir com diversos compostos biológicos e diferentes tipos celulares após sua internalização celular (COHEN et al., 2014).

Com a produção em escala industrial, nanomateriais de carbono podem entrar no ambiente e, devido às suas propriedades químicas, reagir com diferentes substâncias, incluindo os poluentes, sendo os ecossistemas naturais expostos tanto aos nanomateriais, de forma isolada, quanto aos produtos de suas interações (KAHRU; DUBOURGUIER, 2010).

Elucidando as possibilidades de interações no ambiente, no Brasil a contaminação por metais nos ambientes aquáticos é uma realidade, sendo corriqueiramente observados crimes ambientais de despejos de resíduos industriais inapropriados os quais, em sua maioria, possuem metais em sua composição (SOUZA et al., 2013; SOUZA; WASSERMAN, 2015). Adicionalmente, além das atividades industriais, o lançamento de efluentes domésticos sem tratamento e percolação das águas superficiais provenientes de áreas cultivadas com adubos químicos e defensivos

agrícolas também contribuem para a entrada de contaminantes nos ambientes aquáticos (LEWINS et al., 2011).

Outra preocupação associada à presença de metais nos ambientes aquáticos está relacionada às características recalcitrantes desses compostos que, devido à sua elevada resistência à degradação química, física e biológica no sistema aquático, podem persistir biodisponíveis por longos períodos (IKEM et al., 2003). Assim, ao permanecer nesses ambientes, os metais têm sua concentração gradualmente aumentada, elevando assim os níveis na água e a possibilidade de absorção pelos organismos aquáticos (ARAI et al., 2007).

Entre os metais, o cádmio (Cd) é um metal não essencial para a biota e comprovadamente tóxico (CHEN et al., 2014; ZHU; CHAN, 2012), que é encontrado em grandes quantidades no meio ambiente como resultado das atividades industriais (BALMURI et al., 2017). Em ambientes aquáticos, o Cd pode ser acumulado na biota e causar diversos efeitos prejudiciais, bem como ser biomagnificado, via cadeia trófica, e chegar ao consumo humano (HAN et al., 2009; OLSVIK et al., 2016). A toxicidade de Cd é baseada na capacidade de mimetizar outros elementos, como o cálcio e zinco (SANDBICHLER; HÖCKNER, 2016), acumulando-se principalmente no fígado e nos rins, causando disfunção renal e óssea e induzindo a carcinogênese por inúmeros mecanismos (LIU et al., 2009; ZHANG et al., 2014).

Campos-García e colaboradores (2015), em um estudo no qual a espécie de peixe *Oreochromis niloticus* foi exposta ao pesticida comercial (carbofuran) e à nanotubos de carbono de paredes múltiplas, verificaram que esta combinação de contaminantes pode aumentar em até cinco vezes a toxicidade do pesticida nos ambientes aquáticos. Este estudo mostrou que os nanotubos no ambiente podem atuar como carreadores de poluentes, afetando a sobrevivência, o metabolismo e o comportamento dos animais. Adicionalmente, também foram encontrados resultados similares de aumento da toxicidade do chumbo após coexposição com nanotubos de carbono de paredes múltiplas oxidados (MARTINEZ et al., 2013).

Neste contexto, tendo em vista o potencial de interação destes nanomateriais com as estruturas biológicas, é necessário a avaliação da toxicidade destes compostos. Aliado a isso, está a grande dificuldade de padronização amostral e a escassez de

informações precisas sobre o comportamento coloidal destes nanomateriais nos sistemas biológicos. Dessa forma, é importante a elaboração de métodos robustos e reprodutíveis para avaliação desses materiais, bem como a elaboração de políticas de regulamentação, padronização e certificação dos mesmos (UMBUZEIRO et al., 2011; MARTINEZ et al., 2014).

Diversos modelos biológicos podem ser utilizados para acessar a toxicidade de substâncias e compostos químicos. Dentre eles, modelos *in vitro* são uma opção muito utilizada, devido à rapidez com que se pode caracterizar as relações entre os nanomateriais e os diferentes efeitos biológicos (COHEN et al., 2014). Nestes sistemas, geralmente são utilizadas células isoladas que têm seu crescimento em condições controladas, podendo ser expostas aos mais variados agentes de interesse. Posteriormente, parâmetros indicadores da toxicidade podem ser avaliados, tais como a inibição do crescimento e respiração celular, produção de radicais livres, análise de lesões ao DNA, além de outras análises de bioquímicas e morfológicas (MARTINEZ; ALVES, 2013).

Células de peixes são constantemente utilizadas para avaliar a presença de contaminantes e a toxicidade de diversas substâncias. Adicionalmente, diferentes linhagens podem ser utilizadas, objetivando resultados rápidos, precisos e adaptados às condições encontradas em cada estudo (FENT, 2001). Dentre os organismos disponíveis, o peixe-zebra (*Danio rerio*) constitui uma espécie modelo de ampla utilização mundial em testes de toxicidade, possuindo diferentes linhagens de células padronizadas e que têm sido comumente utilizadas pela comunidade científica (FENT, 2001).

Por fim, os efeitos da presença de nanomateriais de carbono e sua interação com metais no ambiente podem ser avaliados com a utilização de diversos biomarcadores, sendo que a integração de análises físicas, químicas e avaliações biológicas representam uma completa e importante abordagem para a avaliação ambiental destes compostos associados.

2 REVISÃO BIBLIOGRÁFICA

2.1 NANOMATERIAIS DE CARBONO

A produção de nanomateriais tem-se intensificado a cada ano, e, devido às suas características químicas e a alta reatividade, estes compostos possuem variadas aplicações (KAHRU; DUBOURGUIER, 2010). Dentre os nanomateriais, as nanoestruturas de carbono se destacam, sendo utilizadas em diversos setores, como na indústria eletrônica e de materiais, em tecnologias voltadas para a saúde, na agricultura e no meio ambiente (VUKOVIC et al., 2011; MATHUR et al., 2017).

Nanomateriais de carbono são alótropos de carbono que possuem uma dimensão de 1 a 100 nm. Existem diferentes classes desses nanomateriais, sendo que os mais conhecidos são os fulerenos, os grafenos, os nanotubos de carbono e o carbono negro (carbono amorfo) (FREIXA et al., 2018). Além destes, outras classes e terminologias são descritas na literatura como as nanocápsulas, os chifres e pontos de carbono quânticos (LIU; LI, 2006; MIYAKO et al., 2008; JALEEL; PRAMOD, 2018).

Os fulerenos são nanomateriais que possuem os seus átomos do carbono na conformação de hexágonos e pentágonos que são unidos e organizados como um poliedro. Esses nanomateriais podem diferir em relação ao número de átomos de carbono presentes na sua estrutura, sendo os mais comumente estudados, os denominados C60 ou C70 (KLAINE et al., 2012).

Os grafenos são materiais constituídos por uma única camada de átomos dispostos como uma rede hexagonal. Essa classe de nanomateriais pode incluir, além do grafeno puro, suas formas reduzidas e oxidadas (ZHAO et al., 2014).

Nanotubos de carbono são compostos formados a partir de uma ou mais camadas de grafeno enroladas, caracterizados por serem materiais com excelentes propriedades de adsorção (MARTINEZ et al., 2014).

Os aspectos relacionados à saúde e à segurança referentes a utilização de nanomateriais de carbono para os seres humanos e para o ambiente ainda não estão bem entendidos e caracterizados, sendo que a avaliação da toxicidade destes materiais

é uma questão importante a ser abordada para o desenvolvimento de uma nanotecnologia segura (MARTINEZ, 2011).

2.2 NANOTUBOS DE CARBONO DE MÚLTIPLAS CAMADAS

Nanotubos de carbono podem ser classificados com base no número de paredes presentes em sua estrutura, como de parede simples, de paredes dupla ou de paredes múltiplas, dependendo do número de paredes cilíndricas e concêntricas que estes materiais apresentem (FREIXA et al., 2018).

Os nanotubos de carbono de paredes múltiplas, do inglês multiwalled carbon nanotubes (MWCNTs), consistem usualmente de 2 a 50 cilindros de carbono com poucos nanômetros de diâmetro empilhados concentricamente com um eixo longo comum (ÖNER et al., 2018; SIEGRIST et al., 2014).

Esses nanomateriais podem passar por um processo de se tornarem funcionais de forma covalente ou não-covalente, gerando grupamentos em suas superfícies que são comumente utilizados para melhorar a dispersão dessas nanoestruturas na água. Adicionalmente, esses grupos funcionais podem ser utilizados para ligar o nanotubo à variados tipos de moléculas, sendo que o tipo de funcionalização mais comum é pelo processo químico de oxidação na presença de ácidos (RAMANATHAN et al. 2005; ALVES et al., 2014).

Devido às características de adsorção, nanotubos de carbono são materiais promissores para tratamentos de águas contaminadas e remediação de poluentes ambientais, como pesticidas, metais, corantes, compostos poliaromáticos e toxinas (MARTINEZ et al., 2013; CAMPOS-GARCIA et al., 2015). Porém, ao serem utilizados para esse fim nos ambientes naturais (intencionalmente ou acidentalmente) poderão provocar impactos adversos.

Os nanotubos de carbono são materiais que estão sendo cada vez mais utilizados em pesquisas biológicas, pelas suas interações com diferentes organismos e estruturas celulares, devido às seguintes características: (a) tamanho nanométrico dessa s estruturas, em dimensões equivalentes a biomacromoléculas e estruturas celulares; (b) capacidade de internalização nas células em geral; (c) possibilidade de

mimetizar compostos e tornar funcional as estruturas internas do nanomaterial e (d) grande potencial de inovação tecnológica (MARTINEZ, 2011).

Apesar de constituírem nanoestruturas cada vez mais utilizadas, o debate acerca da padronização, regulamentação e certificação para os nanotubos de carbono ainda precisa ser realizado, devido, principalmente aos seguintes motivos: (a) a exponencial produção industrial em larga escala e o aumento do risco e exposição para os ecossistemas naturais; (b) a alta reatividade química dessas estruturas e área superficial elevada; (c) a enorme diversidade de composição e estrutura desses materiais; (d) a ausência de adaptação dos estudos toxicológicos tradicionais para os nanomateriais e a carência de trabalhos integrados sobre a temática (MARTINEZ, 2011).

2.3 INTERAÇÃO DE NANOMATERIAS DE CARBONO E CONTAMINANTES AQUÁTICOS

O estudo do comportamento e das transformações de agentes químicos e fatores abióticos no ambiente, bem como os seus efeitos sobre a biota residente é denominado ecotoxicologia. Nesse sentido, a ecotoxicidade ocorre pela interação de vários agentes presentes em um determinado ambiente. Assim, uma avaliação ecotoxicológica deve considerar as fontes de emissão de poluentes, suas interações, transformações e difusões (ZAGATTO; BERTOLETTI, 2006).

Ao atingirem um corpo hídrico, os contaminantes estão sujeitos a influência de outros agentes, que podem levar a sua concentração ou diluição. Estes compostos também podem ser modificados em função das diversas reações químicas que estarão sujeitos no ambiente, bem como as interações com a comunidade biológica local (ZAGATTO; BERTOLETTI, 2006).

Atualmente, evidências sobre os efeitos ecotoxicológicos dos nanomateriais de carbono em organismos aquáticos, bem como a sua disponibilidade para interagir com outros micro-poluentes através da influência da toxicidade e biodisponibilidade de poluentes co-existentes ainda são escassas (MARTINEZ; ALVES, 2013).

Com a produção em escala industrial, nanomateriais de carbono podem entrar nos ambientes e, devido as suas propriedades químicas, reagir com diferentes substâncias, incluindo os poluentes que podem apresentar propriedades tóxicas distintas (KAHRU; DUBOURGUIER, 2010). Por possuir tamanho em escala nanométrica, estes materiais têm a capacidade de penetração celular, podendo interagir com diversos compostos biológicos e diferentes tipos celulares (COHEN et al., 2014).

Um fator primordial para entender os nanomateriais é a realização da caracterização física a química dos mesmos, considerando-os como sistemas complexos que precisam ser investigados de acordo com seu tamanho, forma, área superficial, composição, pureza, dispersão, solubilidade e cargas superficiais, entre outras características, direta ou indiretamente relacionadas ao seu mecanismo de ação nos organismos. Além disso, diferente de uma molécula química comum, os nanomateriais precisam ser estudados em cada detalhe, pois suas características específicas podem estar diretamente relacionados à sua ação no sistema biológico (MARTINEZ; ALVES, 2013).

Neste contexto, tendo em vista o potencial de interação destes nanomateriais com as estruturas biológicas, há uma grande preocupação em relação a seus efeitos tóxicos para o ambiente. Por outro lado, devido à grande dificuldade de padronização amostral, a avaliação de ecotoxicidade destes compostos é crítica. Assim, informações precisas sobre o comportamento coloidal destes nanomateriais são necessárias, por meio da elaboração de métodos robustos e reprodutíveis para estes compostos (UMBUZEIRO et al., 2011; MARTINEZ et al., 2014).

2.4 CÁDMIO

Em relação aos metais no ambiente, para que um metal exiba uma resposta tóxica, é necessário que este esteja biodisponível para ligar-se as superfícies celulares. Em um sistema em equilíbrio, a atividade do íon metálico livre reflete a reatividade química do metal, é essa reatividade que determina a extensão das reações do metal

com os sítios celulares da superfície e, portanto, sua biodisponibilidade. Assim, a quantidade de metal livre deve ser proporcional à sua resposta tóxica (McGEER et al., 2012).

O cádmio (Cd) é um metal cinza claro maleável, cuja superfície escurece em contato com o ar devido ao processo de oxidação (TERRY; STONE, 2002). As fontes naturais de cádmio incluem o intemperismo de rochas (particularmente das rochas fosfáticas), atividades vulcânicas, poeira levada pelo vento e incêndios em florestas. Por outro lado, as fontes antropogênicas estão relacionadas à mineração e refino de minérios de Zn, Pb e Cu, ao uso de fertilizantes fosfatados, à queima de combustível fóssil, turfa e madeira e à fabricação de cimento (McGEER et al., 2012).

O cádmio usualmente apresenta concentrações pequenas no ambiente. Porém, ao passar por processos industriais de extração e enriquecimento, esse metal pode chegar aos ecossistemas naturais em maiores concentrações e causar, assim, danos a biota residente. As principais utilizações do cádmio na indústria são a produção de baterias de níquel e cádmio, dispositivos fotovoltaicos, pigmentos, estabilizadores, revestimentos, resíduos e efluentes industriais e domésticos e ligas metálicas (PAN et al., 2010). Atualmente, a utilização de Cd em nanomateriais, como telureto de cádmio (CdTe) ou selênio de cádmio (CdSe), é uma realidade crescente devido as suas propriedades ópticas (MO et al., 2017).

O cádmio é um metal não essencial e a sua toxicidade nas espécies aquáticas depende da sua biodisponibilidade e sua concentração como íon (Cd^{2+}). No entanto, a partir da complexação com ânions orgânicos e inorgânicos presentes no ambiente ou por meio de interações competitivas com o íons, como cálcio (Ca^{2+}), a toxicidade desse metal pode ser reduzida (McGEER et al., 2012).

Exposições crônicas desse metal levam a distúrbios no crescimento e na regulação iônica dos animais, além de problemas na reprodução, no sistema imunológico, no desenvolvimento endócrino e no comportamento. Além disso, histopatologias nas brânquias, fígado e rins também são induzidas pela presença desse contaminante (McGEER et al., 2012).

O cádmio pode se acumular na biota, tanto do solo para as plantas e organismos terrestres, quanto da água para as espécies aquáticas, sendo o fígado e o

rim os principais órgãos de bioacumulação (ZHU; CHAN, 2012). Este metal também está associado a danos oxidativos, e diversos processos de detoxificação estão envolvidos nos mecanismos de defesa dos organismos, como as enzimas catalase e glutationa peroxidase e a presença de proteínas como as metalotioneínas e a glutationa (McGEER et al., 2012).

Em animais, a meia-vida do cádmio é longa, compreendendo quase 30 anos em humanos, sendo que sua toxicidade aguda e crônica está usualmente associadas à disfunção renal. Nos organismos, o cádmio é complexado com as proteínas metalotioneínas (MT) formando um complexo Cd-MT que é retido ao chegar nos túbulos renais, causando disfunções em pacientes ou animais contaminados (ZHU; CHAN, 2012). A acumulação do cádmio no fígado também é observada em alguns estudos, mas o completo mecanismo dos seus efeitos tóxicos em células hepáticas ainda está sendo investigado (ZHU; CHAN, 2012, CHEN et al., 2014).

O cádmio ocorre naturalmente em baixas concentrações nos ecossistemas aquáticos. Na água doce, o Cd total dissolvido é geralmente menor que 0,5 μg L^{-1} e é ainda mais baixo na água do mar (0,02 μg L^{-1}) (PAN et al., 2010). Porém, recentes estudos relataram níveis de Cd em águas superficiais, com concentrações variando de 5,29 a 27,45 μg L^{-1} em Bangladesh (ALI et al., 2016), de 8,0 a 14,5 μg L^{-1} na Polônia (WOJTKOWSKA et al., 2016) e de 0,20 a 5,89 μg L^{-1} na China (LI; ZHANG, 2010).

A contaminação de cádmio mais comum nos ecossistemas aquáticos ocorre pela disposição inadequada dos resíduos sólidos e pelo derramamento de efluentes contaminados com este metal (CHASIN; CARDOSO, 2003). Os níveis de Cd em áreas poluídas na área norte da China chegam a 4.500 μg L^{-1} no rio Guangdong e 1.120 a 4.474 μg L^{-1} no rio Luan (LI; LIU, 2009).

No Brasil, o a concentração de Cd total permitido nas águas doces varia entre 1 μg L^{-1} (para águas da Classe 1) a 10 μg L^{-1} (para águas da Classe 3) (CONAMA, 2005) e o permitido para despejo de efluentes é de 20 μg L^{-1} (CONAMA, 2011). Em São Paulo, no Rio Pardo, foram reportados valores máximos de Cd de 1,8 μg L^{-1} na água e 0,28 mg Kg^{-1} no sedimento (MACHADO et al., 2016) e nos rios Tietê e Pinheiros o valor máximo encontrado nos sedimentos foi de 6,9 ± 0,4 mg Kg^{-1} (SILVA et al., 2002). Em Salvador, Ferreira e colaboradores (2004) reportaram valores entre 0,035 a 0,17 μg L^{-1}

para a água do mar e no Rio Grande do Norte, no estuário do Rio Jundiaí, os valores foram de 2 a 5 μg L^{-1} (GURGEL et al., 2016).

2.5 PEIXE-ZEBRA (*Danio rerio*)

O peixe-zebra (*zebrafish)*, espécie *Danio rerio* (Hamilton, 1822), pertence à família Cyprinidae. Este animal, quando adulto, apresenta um comprimento médio de 3 a 4 centímetros e exibe um dimorfismo sexual perceptível. Esse peixe, também conhecido como paulistinha no Brasil, e originário do sul da Ásia, foi ao longo dos anos introduzido em diversos ecossistemas, sendo atualmente encontrado em diversos países (ABNT, 2011). Atualmente, é uma das poucas espécies que possui seu genoma inteiramente sequenciado, com 26.206 genes, possuindo 70% de similaridade com o genoma humano (SIEBEL et al., 2015).

O peixe-zebra é um modelo de ampla utilização para a ciência mundial, desde trabalhos com células tronco até estudos de mudanças comportamentais, existindo assim um grande número de estudos comparativos que facilitam a compreensão de muitos mecanismos biológicos do organismo (EIDE et al., 2014). Além disso, são úteis para estudos de toxicologia, pois possuem grande sensibilidade quando expostos à produtos químicos, pela característica de absorver com rapidez os compostos presentes na água e de acumulá-los em diversos tecidos (ZHENG et al., 2016).

Biomarcadores específicos do peixe-zebra também são úteis para avaliar os níveis toxicológicos de poluentes, como a avaliação da concentração letal, efeitos na taxa reprodutiva e aos sistemas de defesa antioxidante, bem como, os danos ao DNA e ao RNA. Dentre os biomarcadores bioquímicos do sistema antioxidante, os mais comumente analisados são a enzima superóxido dismutase (SOD), a catalase (CAT), a glutationa peroxidase (GPx) e o conteúdo de glutationa (GSH), que são os principais componentes do sistema de defesa contra os danos causados pelos radicais livres (GAO et al., 2018).

Células de peixes são constantemente utilizadas para avaliar os efeitos dos contaminantes e a toxicidade de diversos poluentes ambientais (MORCILLO et al.,

2016; BAYOUMI et al., 1999). A espécie *D. rerio* possui diferentes linhagens de células padronizadas que são amplamente usadas em ensaios toxicológicos pela comunidade científica (FENT, 2001). Dentre as linhagens disponíveis, as células de fígado ZFL (do inglês, *zebra fish liver*) possuem uma característica e morfologia geral de hepatócito, sendo úteis em investigações toxicológicas e de mecanismos de desintoxicação, permitindo uma interpretação precisa dos riscos potenciais dos poluentes (CHEN et al., 2014).

As células ZFL são células normais de indivíduos adultos, sendo muito utilizadas em estudos de toxicologia, por apresentarem metabolismo de células hepáticas e possibilidades para investigação dos efeitos dos xenobióticos (GHOSH, 1994). Para evidenciar as características das células ZFL, nas Figuras 1 e 2 são mostradas a morfologia de células em cultivo, observadas em microscópio de luz e duas células isoladas por microscopia eletrônica de transmissão.

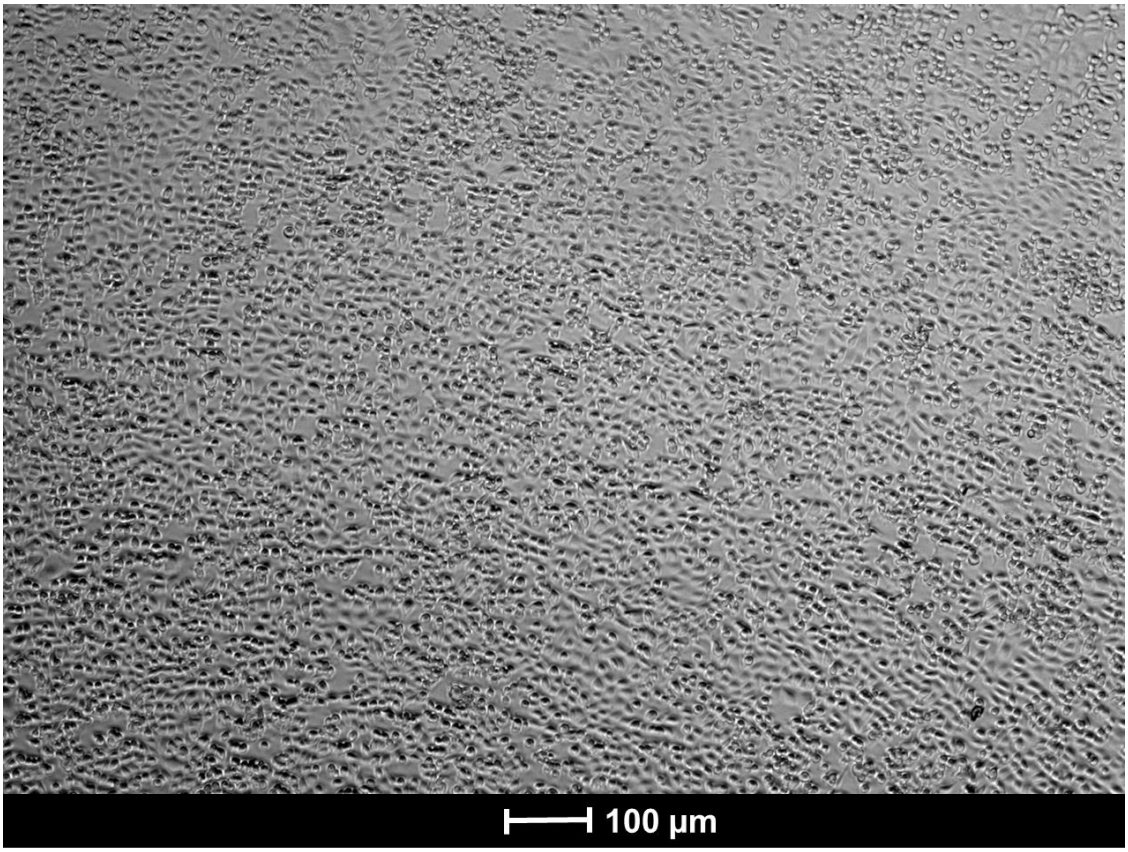


Figura 1 - Imagem das células ZFL em cultivo celular. FONTE: elaborado pela autora.

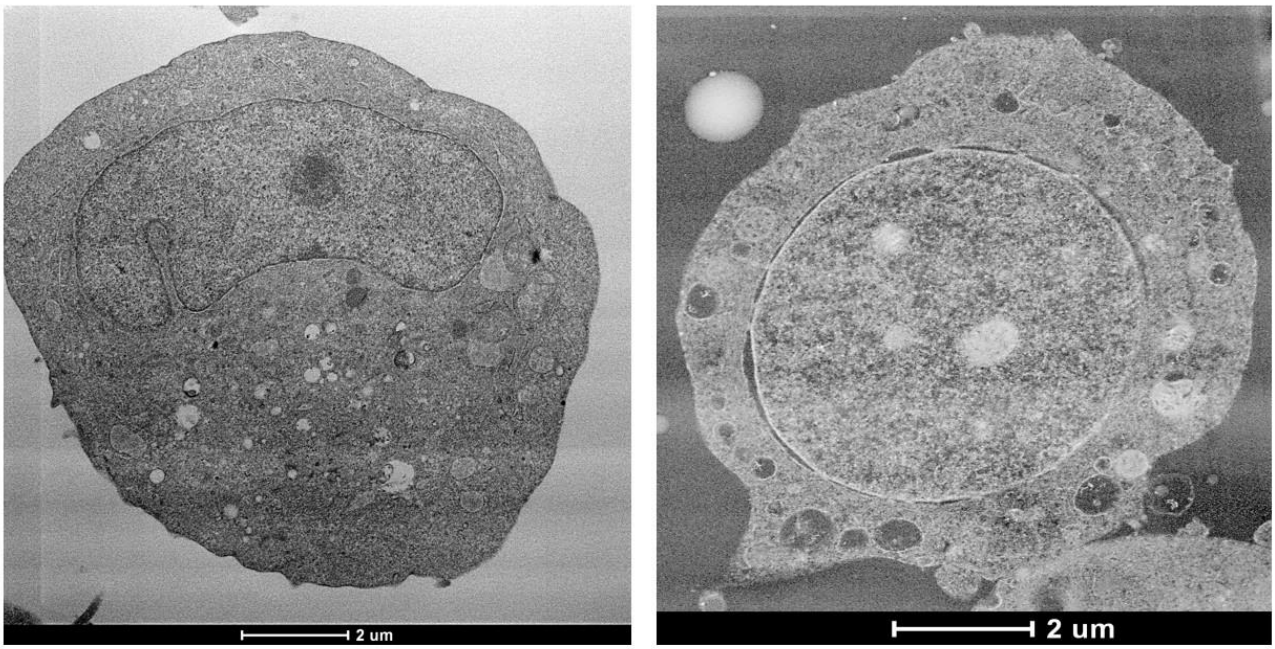


Figura 2 - Imagens das células ZFL por microscopia eletrônica de transmissão.
FONTE: elaborado pela autora.

2.6 ESTUDOS TOXICOLÓGICOS *IN VITRO*

Diversos modelos biológicos podem ser utilizados para acessar a toxicidade de substâncias e compostos químicos. Dentre eles, modelos *in vitro* são uma opção, devido à rápida capacidade de caracterizar as relações entre os nanomateriais e diferentes efeitos biológicos (COHEN et al., 2014). Além disso, são amplamente utilizadas para avaliar os efeitos de misturas complexas de poluentes (STADNICKA-MICHALAK et al., 2014), como mecanismos de captação e sensibilidade celular a agentes químicos e físicos (BONOMO et al., 2016; RAFFA et al., 2010).

Em modelos *in vitro*, geralmente são utilizadas células isoladas que têm seu crescimento em condições controladas e que são expostas aos agentes de interesse. Posteriormente, parâmetros indicadores da toxicidade são avaliados tais como a inibição do crescimento e respiração celular, a produção de radicais livres, análise de lesões ao DNA, além de outras análises de bioquímicas e morfológicas (MARTINEZ; ALVES, 2013).

Em relação aos nanomateriais, para avaliar os efeitos destes em testes *in vitro*, é importante tomar algumas precauções. Na presença de proteínas do soro, necessário para o cultivo e tratamento das células, as superfícies dos nanomateriais são cobertas por uma “coroa de proteínas” formada por múltiplas proteínas que podem influenciar a toxicidade do material estudado e modificar as interações da membrana, acúmulo, mecanismo de endocitose e reações de estresse oxidativo (HUSSAIN et al. 2015; SHARMA et al., 2014; WINZEN et al., 2015). Portanto, essa coroa de proteínas torna-se a interface entre o nanomaterial e as células, e sua análise é essencial para entender seu papel em estudos de toxicidade *in vitro*.

3 OBJETIVOS

3.1 OBJETIVO GERAL

O objetivo geral do trabalho foi avaliar a interação de nanotubos de carbono de paredes múltiplas oxidados (ox-NTCPM) e íons de cádmio (Cd^{2+}) e seus efeitos em células de fígado de *Danio rerio* (linhagem ZFL), utilizando análises *in vitro*, a fim de contribuir com a elaboração e padronização de métodos reprodutíveis e eficazes para a avaliação toxicológica destes compostos.

3.2 OBJETIVOS ESPECÍFICOS

Os objetivos específicos do trabalho foram:

a. Caracterizar quimicamente os nanotubos de carbono oxidados e suas interações com o cloreto de cádmio ($CdCl_2$);
b. Avaliar o potencial de adsorção de cádmio dos nanotubos de carbono oxidados de paredes múltiplas;
c. Verificar a toxicidade dos nanotubos de carbono oxidados de paredes múltiplas, e do cloreto de cádmio isoladamente e de suas interações em células de fígado de peixe-zebra (linhagem ZFL);
d. Avaliar os efeitos da exposição à células de fígado de peixe-zebra (linhagem ZFL) de nanotubos de carbono oxidados de paredes múltiplas, do cloreto de cádmio e suas interações, por meio de análises bioquímicas e citogenéticas.

4 RESULTADOS

4.1 ARTIGO I

Este artigo científico foi submetido e aceito para publicação na revista **Aquatic Toxicology** [ISSN 0166-445X (impressa), 1879-1514 (online)] e já se encontra disponível online (doi: 10.1016/j.aquatox.2018.05.002).

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

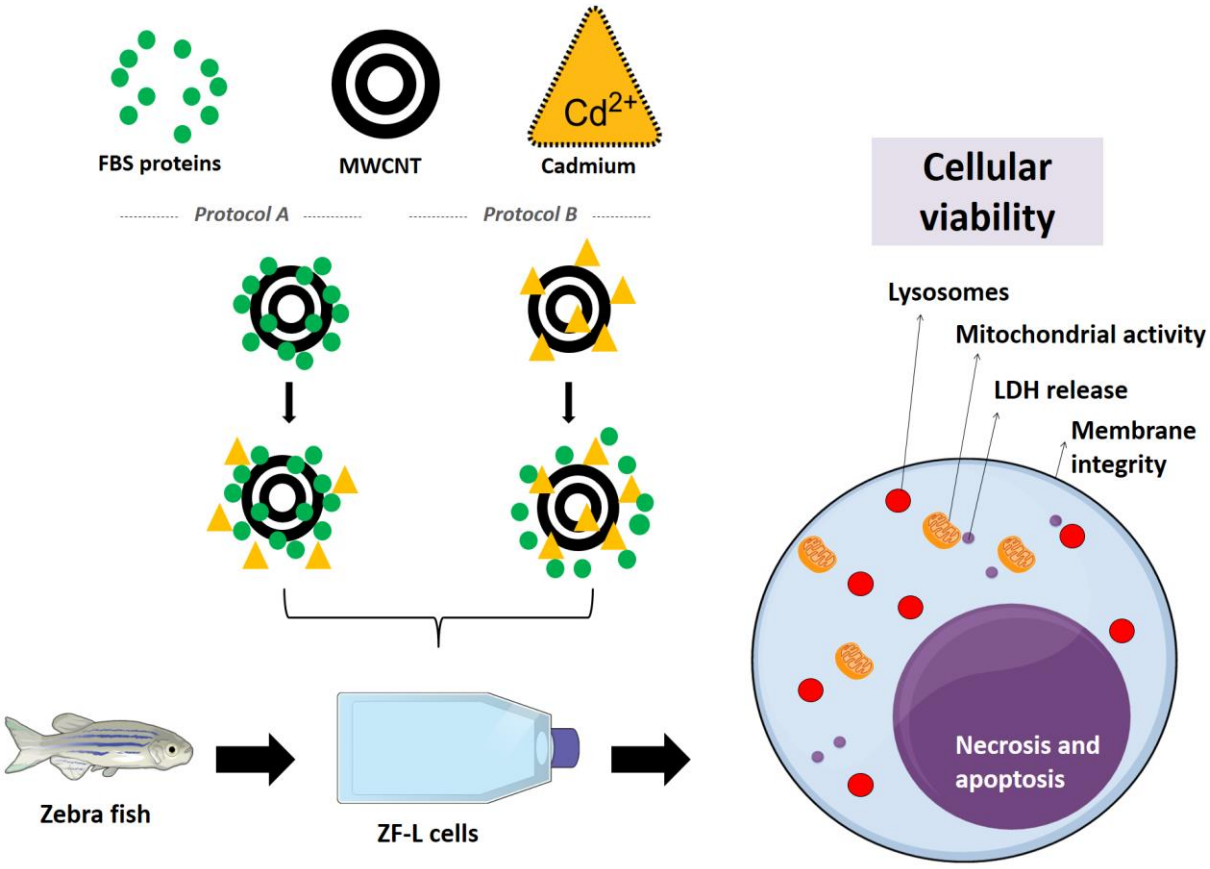
Abstract

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

stability and affects the *in vitro* toxicological assays. Considering that Protocol A showed more ox-MWCNT stability after interaction with Cd, this protocol is recommended to be adopted in future studies.

Keywords: Nanoecotoxicology, protein corona, colloidal stability, cell viability, flow cytometer, cadmium adsorption.

Graphical abstract



1. Introduction

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

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

Carbon nanotubes (CNTs) have numerous applications in industrial, agriculture and biomedical sectors (Freixa et al., 2018; Umbuzeiro et al., 2011; Smajda et al., 2010). Previous studies have already predicted relevant concentrations of these NMs in the environment in the future years due to increasing application demand or accidental circumstances (Keller and Lazareva, 2014; Mueller and Nowack, 2008). In these studies, the predicted concentrations for CNTs in effluents are 0.005 to 0.05 μg L^{-1} and

0.05 to 5 mg kg^{-1} in biosolids by Keller and Lazareva (2014) and 0.05 to 0.1 µg L^{-1} by Gottschalk and collaborators (2013).

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

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

accumulates primarily in the liver and kidneys, causing kidney and bone dysfunction and inducing carcinogenesis by numerous mechanisms (Zhang et al., 2014; Liu et al., 2009).

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

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

It is evident that CNTs are important potential pollutants and Cd is considered one of the most toxic heavy metals for aquatic biota (Ji et al., 2016). Both contaminants are associated with a wide variety of applications, such as electronic engineering, photovoltaic devices, automotive industry (Freixa et al., 2018; Chen et al., 2014). In this

context, this study evaluated the effects of the interaction of ox-MWCNTs with Cd in a zebrafish liver cell line (ZFL). Additionally, we aimed to evidence the influence of the order of carbon nanotube and metal interaction method towards co-exposure toxicity studies.

2. Materials and Methods

2.1. Preparation and characterization of oxidized multiwalled carbon nanotubes

Industrial grade multiwalled carbon nanotubes (MWCNT) were obtained from CNT Co. Ltd. (Incheon, Korea). Oxidized MWCNT (ox-MWCNT) was obtained by dispersing 1.0 g of MWCNT in nitric acid (9 M) and stirring for 12 h, under reflux, at 150 ºC. Then, they were filtered, washed repeatedly with ultrapure water until a neutral pH was obtained and dried for 24 h using a lyophilizer (Enterprise II, Terroni, Brazil) at -48 ºC. Finally, the ox-MWCNT was autoclaved and dispersed in ultrapure water (0.5 mg mL^{-1}) by sonication for 60 min (Ultrasonic Bath, Cole-Parmer 08895-43, USA).

Thereafter, MWCNT and ox-MWCNT thermal stability was evaluated by thermogravimetric analysis (TGA) (STA, 449 F3 Jupiter@, NETZSCH, Germany) employing a heating rate of 5 ºC min^{-1}, from room temperature to 750 ºC, with a synthetic air flow of 50 mL min^{-1}. The structure and morphology of ox-MWCNT was observed by scanning electron microscopy (SEM) (MEV-EDS Inspect F50, FEI, UK) and transmission electron microscopy (TEM) (JEM-1400 plus, JEOL, USA) techniques. Chemical surface analyses were carried out on a K-Alpha photoelectron spectrometer

(XPS) system (Thermo Fisher Scientific, UK) employing pass energies of 200 and 50 eV to obtain the survey and high-resolution spectra, respectively, and data were analyzed using Thermo Avantage software (Version 5.921) and the total oxidation degree was calculated according to Padovani et al. (2015). The hydrodynamic sizes and zeta potentials were evaluated by dynamic light scattering (DLS) and electrophoretic light scattering (ELS) (Zetasizer Nano-Instrument, Malvern, UK), respectively.

2.2. Ox-MWCNT and cadmium co-exposure protocols

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

2.3. Determination of cadmium adsorption by ox-MWCNT

Cadmium adsorption experiments were performed at 25 μg mL^{-1} of $CdCl_2$ and increasing concentrations of ox-MWCNT (0, 5, 10, 25, 50, 100 μg mL^{-1}) in order of each protocol A and B. After the incubation time the medium were centrifuged at 14000 G and

the supernatants were removed. Aliquots of 1 mL of supernatant were digested in triplicate in digester units using 4 mL of distilled HNO_3 at 100 ºC for 12 h in a ceramic plate. Subsequently, the digested content was filtered at 45 µm, weighted and diluted up to twenty times with ultrapure water for analysis. Measurements of Cd content was performed using an inductively coupled plasma optical emission spectrometer (ICP-OES, iCAP 7000 Series, Thermo Scientific) with argon (99.9996%, White Martins-Praxair, SP, Brazil). Experiments were performed using HNO_3 (65%, Merck, Kenilworth, NJ, Brazil) and ultrapure water, resistivity higher than 18.5 MΩ cm, (Gehaka, Sao Paulo, Brazil).

Controls of method were prepared using the same protocol without sample (only reagents) in triplicate. Standard solutions used for ICP-OES calibration and for addition and recovery experiments were prepared by dilution of 1000 mg L^{-1} of Cd in 0.14 mol L^{-1} of HNO_3. The concentrations of the solutions used for obtaining analytical calibration curves were 0, 25, 50, 75, 100, 125, 150, 175 and 200 µg L^{-1}. The detection limits (LOD) was calculated considering standard deviation (SD) for 10 measurements of a blank solution (Thomsen et al., 2003). The accuracy of the ICP-OES analytical procedure was evaluated by addition and recovery experiments. The recovery and relative standard deviation obtained for Cd was 96.3±6.6%. The LOD and LOQ for ICP-OES analysis were 0.12 and 0.35 µg L^{-1}, respectively.

2.4. Dispersion characterization and colloidal stability studies

The colloidal stability of 1, 10, 25 and 50 μg mL^{-1} ox-MWCNT in RPMI/L-15 cell culture medium, with and without the addition of 10% FBS, were evaluated by measure the optical density at 400 nm (UV-Vis spectrophotometer, Multiskan GO, Thermo Scientific, UK) at 0, 1 and 24 h. The dispersion stability of complexes formed from Protocols A and B, at different ox-MWCNT concentrations and fixed 25 μg mL^{-1} of $CdCl_2$, were also monitored. The stability studies were performed in triplicate. The concentrations set for this study was chosen based in previous studies (Sanchís et al., 2016; Campos-Garcia et al., 2015; Martinez et al., 2013) and previous experimental tests in the laboratory (data not shown).

Bare ox-MWCNT and the intermediates and final complexes of ox-MWCNTs with FBS proteins and $CdCl_2$, obtained from Protocols A and B, were characterized by DLS and ELS, respectively. For particle size (hydrodynamic diameter) and surface charge (zeta potential) measurements, bare ox-MWCNT was dispersed in ultrapure water at 10 μg mL^{-1} and, the intermediates and final complexes of ox-MWCNTs with FBS proteins and $CdCl_2$, obtained from Protocols A and B, were washed three times with phosphate buffer (PBS), in order to remove the unbound proteins and Cd excess, and ressuspended in ultrapure water at 10 μg mL^{-1}.

2.5. Protein corona assay

Complexes from Protocols A and B, obtained after incubation of 25 μg mL^{-1} of ox-MWCNT and 25 μg mL^{-1} of $CdCl_2$ in RPMI/L15 medium + 10% FBS for 1h at 37°C, were washed three times with PBS to remove the weakly bound FBS proteins on the

nanotube surface (soft corona). After washing, the final pellets were comprised of the ox-MWCNT covered by strongly adsorbed FBS proteins (hard corona). A control with only ox-MWCNT, in the absence of $CdCl_2$, was also performed. The hard corona proteins were extracted from nanotube surface using loading buffer (New England BioLabs, Ipswich, USA), and 15 µL of the protein solution were loaded in 15% SDS polyacrylamide gel electrophoresis (SDS-PAGE). The protein bands contrast in the gels were enhanced by silver staining. All experiments were performed in independent triplicates.

2.6. *In vitro* toxicity assays

2.6.1. ZFL cell line culture conditions

The liver cell line of zebrafish (*Danio rerio*) (ZFL) was provided by Rio de Janeiro Cell Bank, Brazil (allotment: 0313) cultivated (passage numbers 9–20) and transferred to 24-well (for trypan blue, lactate dehydrogenase (LDH) and apoptosis assays) and 96-well (for MTT and neutral red assays) plates with RPMI/L-15 culture medium (Cultilab, Brazil), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, at $4x10^5$ cell/well and $2.5x10^5$ cell/well concentrations, respectively. Plates were maintained in a BOD type incubator at 28 ºC without carbon dioxide.

2.6.2. Cell treatment

After stabilization, cells were exposed for 24 h at 28 ºC, to the following treatments: (I) negative control, culture medium supplemented with FBS; (II) ox-MWCNTs, culture medium supplemented with FBS and 10 µg mL^{-1} ox-MWCNTs; (III) Cd treatments, 5, 10, 15, 20 and 25 µg mL^{-1} of $CdCl_2$ in culture medium supplemented with FBS; (IV) Incubation Protocol A, culture medium supplemented with FBS and 10 µg mL^{-1} of ox-MWCNT for 30 min and subsequent addition of 5, 10, 15, 20 and 25 µg mL^{-1} of $CdCl_2$ for more 30 min; and (V) Incubation Protocol B, 10 µg mL^{-1} of ox-MWCNT and 5, 10, 15, 20 and 25 µg mL^{-1} of $CdCl_2$ for 30 min and, subsequent addition of culture medium supplemented with FBS for more 30 min. Based on molecular weight of cadmium chloride (183.31 g mol^{-1}, purity 99.3%), the calculated Cd molarity of each concentration used was 27.47, 54.93, 82.40, 109.87 and 137.34 µ mol^{-1}.

2.6.3. Cell viability assays

For each viability assay, an additional experimental control was performed with only ox-MWCNT in cell culture medium (RPMI/L15) with FBS and in the absence of cells in the presence of test reagent to verify the interference of ox-MWCNT with cytotoxicity tests.

2.6.3.1. MTT assay

The MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was performed as described by Mosmann (1983). At the end of treatment period, cells

were washed with PBS three times and incubated with MTT salt (0.05 g mL^{-1}) for 4 h. Thereafter, 200 µL of DMSO was added to each well in order to dissolve the precipitated formazan. The solution was mixed and the absorbance of each well was read in a Dynex microplate reader (Dynex, MRX TC, USA) at 540 nm.

2.6.3.2. Trypan blue® exclusion test

The trypan blue dye exclusion test was performed according to Strober (2001). After treatments, cells were washed with PBS three times, detached from well plate and 10 µL of cell suspension in a new media were mixed with 10 µL of trypan blue solution (GIBCO®, UK). This mixture was added to a cell counter chamber and the number of viable and nonviable cells was determined by an automated cell counter (TC20®, Bio-rad, USA). The test was performed three times and in duplicate per treatment. Images of culture confluence were also recorded with a light microscope (ZEISS, AX10, Germany).

2.6.3.3. Neutral red assay

Neutral red uptake assay for the estimation of cell viability/cytotoxicity was performed according to Repetto et al. (2008). After treatment, cells were washed with PBS three times and incubated with 100 µL of neutral red solution (40 µg mL^{-1}) for 4 h. Thereafter, the cells were washed with 150 µL PBS and 200 µL distain solution (50% ethanol 96%, 49% deionized water, 1% glacial acetic acid) was added into each well.

The plates were shaken for 10 min and the absorbance of each well was read in a Dynex microplate reader (Dynex, MRX TC, USA) at 540 nm.

2.6.3.4. LDH assay

The lactate dehydrogenase assay was performed according to Han et al. (2011). After treatment, the plates were shaken briefly to homogenize the released LDH in the medium; thereafter, the medium was transferred to 1 mL microcentrifuge tubes and centrifuged at 12,000 × g and 4 ºC for 15 min to remove cell fragments and ox-MWCNTs. In this assay, Triton X-100 (0.5% v v^{-1}) (Sigma-Aldrich, USA) was used as a positive control. A volume of 40 µL of each sample of medium was added to 260 µL of substrate solution and the absorbance at 340 nm was measured using in a Dynex microplate reader (Dynex, MRX TC, USA) for 10 min. The LDH activity was obtained by measuring the decreasing rate of NADH absorbance over time (slopes).

2.6.4. Flow cytometer analysis

The apoptotic/necrotic rates were determined with an Annexin V Apoptosis FITC Detection kit (BD Bioscience, NJ, USA) in ZFL cells treated with 5, 10 and 15 µg mL^{-1} of $CdCl_2$ for both protocols, negative control and ox-MWCNT control. The cells were incubated in AnnexinV-FITC and a propidium iodide (PI) labeling solution for 30 min; after staining, they were washed and analyzed using a BD Accuri C6 Flow Cytometer

(BD Bioscience, NJ, USA). The data were obtained based on the FL3 and FL2 channels.

2.7. Statistical analysis

Data were tested by Shapiro-Wilk normality test and treatments were compared using ANOVA followed by a Tukey post hoc test ($p<0.05$), when applicable, or a nonparametric Kruskal-Wallis test ($p < 0.05$) using Infostat software (Di Rienzo et al., 2010). Each experimental condition was performed in biological culture triplicate and technical replicates.

3. Results

3.1. Characterization of oxidized multiwalled carbon nanotubes

The ox-MWCNT prepared in this study was characterized by several techniques prior to cytotoxicity evaluation. The representative morphology (SEM and TEM images) of ox-MWCNT was preserved after sterilization (Fig. 1). The decomposition temperature obtained by TGA analysis was from 500 to 750 ºC for both MWCNT and ox-MWCNT; however, the ox-MWCNT presented a weight loss about 8% at temperatures below that 500°C (Fig. 2A). By XPS survey analysis, only carbon and oxygen atoms were detected, with an oxygen content about 6.89% (Table 1). High resolution XPS measurements (Fig. 2B) provided detailed information of the functional groups present on the ox-MWCNT

surface. Fitting results of C1s showed the presence of C–O, C=O and COO bonds at 286.7, 288.0 and 289.2 eV, respectively, in addition to C=C and C–C bonds at 284.6 and 285.6 eV and the total oxidation degree (DOxid) was 0.15.

3.2. Interaction of ox-MWCNT with cadmium and FBS proteins

By monitoring the dispersion stability of ox-MWCNT in RPMI/L15 medium with and without FBS (Fig. 3A), it is confirmed that ox-MWCNT was stable in RPMI/L15 medium supplemented with FBS. However, in the absence of FBS, the ox-MWCNT precipitated after 24 h. Therefore, culture media supplemented with FBS was used for all treatments for cytotoxicity assessment. In the dispersion stability study of the two co-exposure protocols (Fig. 3B), it was observed that complexes from Protocol B were less stable than complexes from Protocol A.

Particle size and zeta potential of bare ox-MWCNTs and intermediate (step 1) and final complexes (step 2) from Protocols A and B characterized by DLS and ELS, respectively, are presented in Table 2. Ox-MWCNT presented a smaller size than both complexes with FBS and Cd, in which complexes from Protocol B showed a higher size than complexes from Protocol A. Zeta potential of ox-MWCNT was changed from -62.8 to about -36.0 mV and -32.0 mV after interaction with cadmium (Protocol B, step 1) and FBS proteins, respectively.

Adsorption percentages of Cd by ox-MWCNT in Protocol A and B were shown in Figure 4. Differences among protocols can be observed in which Protocol B present higher values of adsorption than Protocol A. At the maximum ox-MWCNT concentration

used (100 μg mL^{-1}) the adsorption values was 11.6±1.5% for Protocol A and 17.7±1.5% for Protocol B. Figure 5 shows the gel electrophoresis of proteins that composes the hard coronas formed on ox-MWCNT in protocols A and B (in the presence of cadmium) and in the absence of cadmium (control); in which it was observed that the protein corona composition was the same for all conditions.

3.3. Toxicity of oxidized multiwalled carbon nanotubes and cadmium

The viability of ZFL cells evaluated by the MTT, trypan blue exclusion test, neutral red and LDH assays are shown in Figure 6 and the cell death analysis by Annexin V and propidium iodide (PI) staining are shown in Figure 7. No differences were observed in cell viability between the negative control and only ox-MWCNT treatment, except for the LDH assay in which cell viability decreased after exposure to ox-MWCNT. However, cell viability showed a dose-dependent response to $CdCl_2$ exposure and after co-exposure ox-MWCNT and $CdCl_2$ (Protocols A and B).

MTT assay revealed that 20 and 25 μg mL^{-1} of $CdCl_2$ are cytotoxic concentrations for ZFL cells in all exposure protocols (Fig. 6A). Trypan blue exclusion test results (Fig. 6B) shown that at low $CdCl_2$ concentrations (5, 10 and 15 μg mL^{-1}) exposure alone, the cell viability in Cd control did not differ from the negative control and ox-MWCNT exposure alone; cell viability decreased significantly in co-exposure with ox-MWCNT and $CdCl_2$ (Protocol A and B). At higher $CdCl_2$ concentrations exposure alone (20 and 25 μg mL^{-1}) the cell viability decreased and did not differ from those from Protocol A and B showing similar responses to the MTT results.

The neutral red assay (Fig. 6C) confirmed the results of the MTT and trypan blue assays in which the treatments with only ox-MWCNT did not reduce cell viability. Only $CdCl_2$ exposure causes cell viability reduction as the metal concentration increased. However, the interaction between ox-MWCNT and $CdCl_2$ showed different responses between Protocols A and B, in which Protocol A treatments showed a similar response to $CdCl_2$ only, while Protocol B treatments presented higher absorbance values in this comparison (Fig. 6C).

For LDH assay, all treatments with ox-MWCNT and $CdCl_2$ shown lower absorbance values compared to the negative control and no differences were observed between them (Fig. 6D). Flow cytometry analysis with Annexin V and propidium iodide (PI) staining (Fig. 7) showed that treatment with only ox-MWCNT induced apoptosis and necrosis while treatments with ox-MWCNT co-exposed with Cd presented very low necrosis rate (~ 1%) and about 6% of apoptotic rates; apoptosis increased at treatment 15 μg mL^{-1} of $CdCl_2$ alone and Protocol A and B. Our experiments with cells exposure confirmed that for the same nanotube and metal concentrations, the order of co-exposure changes nanotube stability, as we observed a higher number of agglomerations on cells in Protocol B (Supplementary information, Fig. 1S).

4. Discussion

The decomposition behavior of ox-MWCNT compared to MWCNT, attest that the oxidation process was successful, once the ox-MWCNT presented a weight loss about 450 ºC, which can be attributed to decomposition of oxygenated functional groups on

the ox-MWCNT surface. The metallic inorganic residues in CNTs can be detected by TGA analysis when their quantity is higher than the detection limit of the equipment (Andrade et al., 2013). By considering the decomposition curve of raw MWCNT, it was observed an amount of inorganic residues about 3.5% at 750 °C, while for ox-MWCNT sample the impurities were not detected (the weight loss reached 100%) in this temperature value. Thus, the oxidation process by nitric acid reduced the ox-MWCNT metallic impurities (i.e. iron oxide). Similar results were also demonstrated by Andrade et al., 2013.

The XPS analysis confirmed the insertion of oxygenated groups on ox-MWCNT surface by oxidation process. The presence of oxygenated groups on the CNT surface is a key point for the interaction of Cd with ox-MWCNT. Oxidized carbon NMs (negatively charged) interact with metal ions, e.g. Cd^{2+}, Pb^{2+}, Cu^{2+}, Zn^{2+} (positively charged), by electrostatic forces. Moreover, the oxygenated groups on the CNT surface are responsible for the improvement of the nanotube water dispersion. Due to these properties, ox-MWCNT are emerging materials for metal removal from water and environmental remediation (Tofighy and Mohammadi, 2011; Lasheen et al., 2015).

When nanomaterials are exposed to biological compounds, such as FBS, they interact with biomolecules from the medium, especially proteins, forming a molecular coating known as “protein corona”. The protein corona is a complex layer of stable and spontaneously formed proteins that cover the NM original surface properties (Peng and Mu, 2016). Thus, the physiological response to ox-MWCNT can be modified by the protein corona formed by FBS proteins, which will be the first surface to interact with cells (Cai et al., 2013).

The presence of proteins in the cell culture medium was also recognized to improve the nanoparticle dispersion stability (Winzen et al., 2015; Sharma et al., 2014; Hussain et al., 2015). The two protocols employed to evaluate ox-MWCNT interaction with $CdCl_2$ in the presence of FBS proteins differed from each other by the interaction order with ox-MWCNT. Carbon nanotubes show exceptional adsorption capacity with divalent metal ions, and several studies have recognized the role of oxygenated functional groups of the CNT surface on the adsorption of metal ions (Cho et al., 2010; Rao et al., 2007; Li et al., 2003; Gao et al., 2008). Therefore, in the Protocol B, the Cd interaction with oxygenated functional groups of ox-MWCNT neutralizes negative charges on the nanotube surface and leads to aggregation; the later FBS proteins interaction with these aggregates form larger complexes, which are less stable and precipitate over time (Fig. 3B, Protocol B). The first interaction of ox-MWCNT with FBS proteins and, then, with $CdCl_2$ maintain the complex formed by ox-MWCNT, FBS and $CdCl_2$ well dispersed as in Protocol A (Fig. 3B, Protocol A).

In particle size and zeta potential analysis the slight increase in size presented by complexes from Protocol A can be attributed to the protein corona formation in the ox-MWCNT surface. However, the complexes from Protocol B have shown a considerable increase in size, which can be attributed to nanotube aggregation due to $CdCl_2$ interaction. These data justify the results found by stability study in which complexes from Protocol B precipitated after 24h. Zeta potential changes after interaction with cadmium (Protocol B, step 1), confirming the role of oxygenated functional groups (negatively charged) on cadmium adsorption by ox-MWCNT. After interaction with FBS

proteins, the zeta potential values refer to the charge of proteins on the nanotube surface, which justifies the similar values found by both final complexes.

A complete study of adsorption of Cd by oxidized carbon nanotubes was previously reported by Akl and Abou-Elanwar (2017) in which the nitric acid-modified carbon nanotubes and bare carbon nanotubes were used. In our study, we showed that order of interactions of metal and nanotube can influence the Cd adsorption by MWCNT in cell cuture media supplemented with FBS. This result can be related to the Cd(II) ability to bind to the ox-MWCNT probably due to the greater availability of exchangeable sites for ions in Protocol B than Protocol A. The adsorption values found (17.7 ± 1.5%) in Protocol B compared with others studies, probably was related with the conditions used for *in vitro* experiments and the low adsorbent dosage used. Akl and Abou-Elanwar (2017) show 100% of Cd adsorption by nitric acid-oxidized MWCNT in water, but pH values higher than 6.5 were not tested and the author's used solutions at 100 ppm of Cd and 16000 ppm of carbon nanotubes. In our study, the experiments were with 15.24±0.013 ppm of Cd and 5 to 100 ppm of MWCNT, at 7.2 pH and a total time interaction of 60 min; these experimental differences probably influenced the results reported.

The cytotoxicity of CNT is attenuated after protein binding, and some proteins show preferential CNT binding, such as peptides rich in the amino acids histidine, tryptophan and phenylalanine (Ge et al., 2011). The protein corona occurs by secondary bonds, like Van der Waals' forces, hydrophobic interactions, electrostatic forces and hydrogen bonds (Peng and Mu, 2016). The CNT affinity with proteins may be influenced by its structure and physicochemical properties, and, once the oxygenated functional

groups on ox-MWCNT surface play a crucial role on the cadmium and proteins adsorption, it was expected that the presence of cadmium could alter the protein corona composition.

The protein corona had the same composition for all conditions, implying that nor cadmium presence neither the order of compounds addition interfered in protein adsorption by ox-MWCNT. These results may be due to the exceptional adsorption capacity of carbon nanotubes and the small amount of cadmium in solution that was not enough to avoid or decrease the protein adsorption.

The toxicity of NMs has been demonstrated in a range of *in vitro* cell viability assays, particularly in mammalian cells (Guadagnini et al., 2015), but only few studies have investigated its effects on fish cell lines (Costa et al., 2012; Ferreira et al., 2014). Azhdarzadeh and collaborators (2015) showed that CNTs can interact and/or modify the properties of almost all toxicity assays, and the explanations for these effects are related to the limitations of each assay and other relevant parameters, such as protein corona, aggregation status, cell shape and exposure conditions, which strongly affect the toxicity responses of NMs. Then, in our study, it is important to notice that five different bioassays were done to assess the toxicity of co-exposure ox-MWCNTs and $CdCl_2$ in an attempt to minimize the possible effect of CNTs interactions or modification of toxicity assay.

Casey and collaborators (2007) showed that the MTT test presented a false negative result when evaluating A549 human cells exposed to single walled CNTs, due to the converted formazan precipitate adsorption by the CNTs. Thus, to exclude the possibility of such experimental artifact in our study, an experimental control was

performed using 10 µg mL^{-1} of ox-MWCNT in the presence of MTT reagent without cells and no significant difference was found between the absorbance of negative control and the ox-MWCNT with the MTT reagent (data not shown). Based on this result and those showed in Figure 6A, in which no differences occurred between negative control and ox-MWCNT treatment, we confirmed that ox-MWCNT at 10 µg mL^{-1} did not interfere in the cell viability results obtained by MTT test.

Cd stimulates the production of reactive oxygen species through different pathways and, consequently, leads to mitochondrial damage (Sandbichler and Höckner, 2016), as occurred in our experiments. Mammalian cell lineages (HTC and HepG2) showed a different EC50 for $CdCl_2$ using the MTT test (100 µM and 15 µM respectively for each cell type for 24h) (Fotakis and Timbrell, 2006), and ZFL cell death were induced after exposure to high (99.6 µM) concentrations of $CdSO_4$ (Tang et al., 2013); both authors did not explain the presence or absence of FBS in their treatments. These results are probably related to Cd concentration and resistance of the cell type analyzed.

The change in cell viability induced by co-exposure ox-MWCNT by trypan blue exclusion test may be explained as a “Trojan horse” action of ox-MWCNT facilitating the entrance of Cd adsorbed to nanomaterials into the cells as hypothesized by Ferreira et al. (2014), Deng et al. (2017); Limbach et al. (2007) or a synergistic effect in which ox-MWCNT can damage the cell membrane by a physical mechanism and lead to cell death. However, the absence of differences between treatments of $CdCl_2$ alone and Protocols A and B at higher $CdCl_2$ concentrations (20 and 25 µg mL^{-1}) may be due to direct action of increasing Cd concentration instead of ox-MWCNT as it concentrations was equal in both protocols independent of $CdCl_2$ concentration.

Neutral red (NR) is a cationic dye that readily penetrates cell membranes by nonionic diffusion and accumulates in lysosomes; damage cells have less capacity to taking up the dye into lysosomes (Casey et al., 2007). SWCNT at very high concentrations (800 μg mL^{-1}) is known to interfere in the dye absorbance (Casey et al., 2007). Then, we also performed an experimental control assay using 10 μg mL^{-1} of ox-MWCNT in the presence of NR reagent without cells and no significant difference was found between the absorbance of negative control and the ox-MWCNT with the NR reagent (data not shown). No significant difference was also found in the absorbance between the negative control and ox-MWCNT treatment with cells (Figure 6C).

The data obtained in the NR assay may be related to the interference of the product from protocol B on the absorbance in this assay in which was observed more ox-MWCNT precipitation on the cells (Fig. 3B, Supplementary information, Fig. 1S). Once protocol B forms large complexes that are not stable in cell culture medium, after 24h of treatment the complexes had precipitated on the cells. When cells were lysed, these complexes return to the suspension and interfere in the absorbance measurement of NR dye released by cells, leading to an increase in the absorbance values. It suggests that the protocol B may induces false viability result by neutral red assay, evidencing that this protocol was not suitable for co-exposure toxicity assessment of MWCNT and Cd.

Another possibility is that Cd can cause lysosomal damage without affecting the cell membrane or mitochondria (Fotakis and Timbrell, 2006). Cd chelation is mediated by glutathione (GSH) and metallothionein (MTs); GSH is considered an essential molecule in the first line of an antioxidant system against xenobiotics and it is required

by GPx and GST activity, but may directly bind metals with its SH group (sulfhydryl) (Pereira et al., 2016); MTs may bind Cd and form MT–Cd complexes in the liver, which are initially stored in lysosomes (Sandbichler and Höckner, 2016).

The uptake of oxidized single-walled nanotubes (ox-SWCNT) was via endocytosis in Jurkat, CHO, HeLa, HL60, NIH 3T3 cells (Kam et al., 2004, 2006; Yaron et al., 2011). Endocytosis consists of engulfment of the cell membrane and formation of a lipid vesicle around the species to be internalized, forming endosomes that may fuse with lysosomes, leading to degradation or inactivation of the internalized species. The formation of CNT conjugates increases the pH inside endosomes resulting in osmotic pressure that induces the enlargement of the endosomal compartments and eventual rupture (Kam and Dai, 2005). Enlarged lysosomes after CNT treatment were also observed in CRND8 glia cells (Xue et al., 2014), and the endocytosis process was observed in ZFL cells (Ruyra et al., 2013; 2015) with the internalization of nanoparticles at only 6 h of treatment (Jiménez-Fernández et al., 2014). Considering that ZFL cells in our experiments did not proliferate due to their long cell cycle (72 h) and short time treatment (24 h), our results may be also related to the increased formation of endosomes and the enlargement of lysosomes resulting in a false viability data in Protocol B.

The LDH assay presented different patterns of response in relation to the other viability tests (Fig. 6D). These results reveal adsorption and consequent inactivation of LDH molecules by ox-MWCNT and an interference of $CdCl_2$, which may interact with LDH and inactivate them. Han et al. (2011) using rat lung epithelial cells showed that several NMs may interfere in the LDH assay by inactivating LDH. In addition, Fotakis

and Timbrel (2006) tested different concentrations of $CdCl_2$ in mammalian cells (HepG2 and HTC) and demonstrated that the LDH assay is less sensitive than other cytotoxicity tests. Our results are in accordance with the literature data and emphasize that LDH assay is not suitable for the assessment of joint toxicity of NMs and Cd, once both of them can inactivate the LDH.

Despite the differences in the colloidal behavior (Supplementary information, Fig. 1S), it was not observed different toxicity responses for complexes from protocols A and B, except for neutral red assay. This result can be attributed to the same composition of the protein corona formed on ox-MWCNT from protocols A and B, as observed by SDS-PAGE analysis (Fig. 5), once the protein corona is the surface that will come to contact with the cell (Walczyk et al., 2010). FBS can influence the metal effects, leading to changes in resistance and/or tolerance of cells, as occurred in SAF-1 (Morcillo et al. 2016), RTG-2 and BF-2 (Bayoumi et al., 1999) and ZFL in the present study, compared with values without FBS reported by Chen and collaborators (2014).

Flow cytometry analysis showed that treatment with only ox-MWCNT increase apoptosis and necrosis rates while co-exposure treatments with ox-MWCNT and Cd presented very low necrosis rate. Contradictory responses were also observed in previous studies in which SWCNTs increase the apoptotic rate in Hep-G2 cells (Kang et al., 2008) and PC12 cells while MWCNTs increase necrosis (Zeinabad et al., 2016). Additionally, Chen and collaborators (2014) reported that Cd induces malignant transformation in ZFL cells, leading to apoptotic resistance, which may be associated with an increase in gene expression, resulting in the stimulation of cell proliferation or apoptosis blockage.

4. Conclusions

This study provides novel insights concerning the interaction of ox-MWCNT with cadmium and FBS proteins towards co-exposure protocols development. Different ox-MWCNT–metal complexes may be formed in the presence or absence of FBS in cell culture medium; furthermore, the order of compounds interactions in co-exposure experiments promotes different ox-MWCNT stability and Cd adsorption rate as observed in protocol A and B. Protocol A in which ox-MWCNT first interacts with FBS and thereafter with metal is recommended to be adopted in future studies as it presented higher ox-MWCNT stability. Treatment with only ox-MWCNT at 10 μg mL^{-1} did not cause toxicity on ZFL cells however, the co-exposure of ox-MWCNT-$CdCl_2$ enhanced toxicity at low $CdCl_2$ concentration (trypan blue assay) suggesting possible “trojan horse” and/or synergistic effect of ox-MWCNT and Cd. Except for neutral red assay results, the absence of significant differences on toxicity of complexes from protocols A and B may be explained by the same protein corona composition observed for both protocols. Improved methods for producing standardized and reproducible protocols during co-exposure studies with environmental pollutants are needed in order to evaluate the cytotoxicity of carbon nanotubes as the potential interactions with existing contaminants are of crucial importance in assessing the biological impacts of CNTs in aquatic organisms.

Conflict of interest statement

The authors declare no conflicts of interest.

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Table 1. Atomic composition of ox-MWCNT and complexes (FBS protein corona coated ox-MWCNT) obtained by protocols A and B, determined by survey XPS.

	Autoclaved ox-MWCNT		Protocol A		Protocol B	
	Atomic %	**SD %**	**Atomic %**	**SD %**	**Atomic %**	**SD %**
C	93.11[a]	0.03	69.35[b]	4.37	74.27[b]	4.47
O	6.89[a]	0.03	15.50[b]	2.22	13.74[b]	2.05
N	--	--	9.42[a]	1.55	9.36[a]	1.30
Cl	--	--	2.73[a]	0.88	1.33[a]	0.34
Na	--	--	2.00[a]	0.60	1.31[a]	0.85
F	--	--	1.00	0.23	--	--

Different letters indicate significant differences (ANOVA, Tukey, $p<0.05$) for the same element.

Table 2. Particle size, polydispersity index (PDI) and zeta potential (ZP) of ox-MWCNT and complexes (FBS protein corona coated ox-MWCNT) obtained by protocols A and B, determined by DLS and ELS, respectively.

Sample		Size ±SD (nm)	PDI	PZ ±SD (mV)
Ox-MWCNT		203.0 ± 3.17	0.42	-62.8 ± 2.15
Protocol A	Step 1	335.0 ± 9.85[a]	0.37	-31.3 ± 0.90[a***]
	Step 2	397.0 ± 16.50[a]	0.36	-32.0 ± 1.13[a***]
Protocol B	Step 1	2720.0 ± 591.60[b***]	0.56	-36.0 ± 0.71[b***]
	Step 2	1022.5 ± 26.20[c*]	0.37	-32.5 ± 0.71[a***]

Different letters indicate significant differences among the protocols (ANOVA, Tukey, $p<0.05$). Asterisk indicates significant differences from the ox-MWCNT (***) $p<0.001$, (**) $p<0.05$, (*) $p<0.01$.

Figures

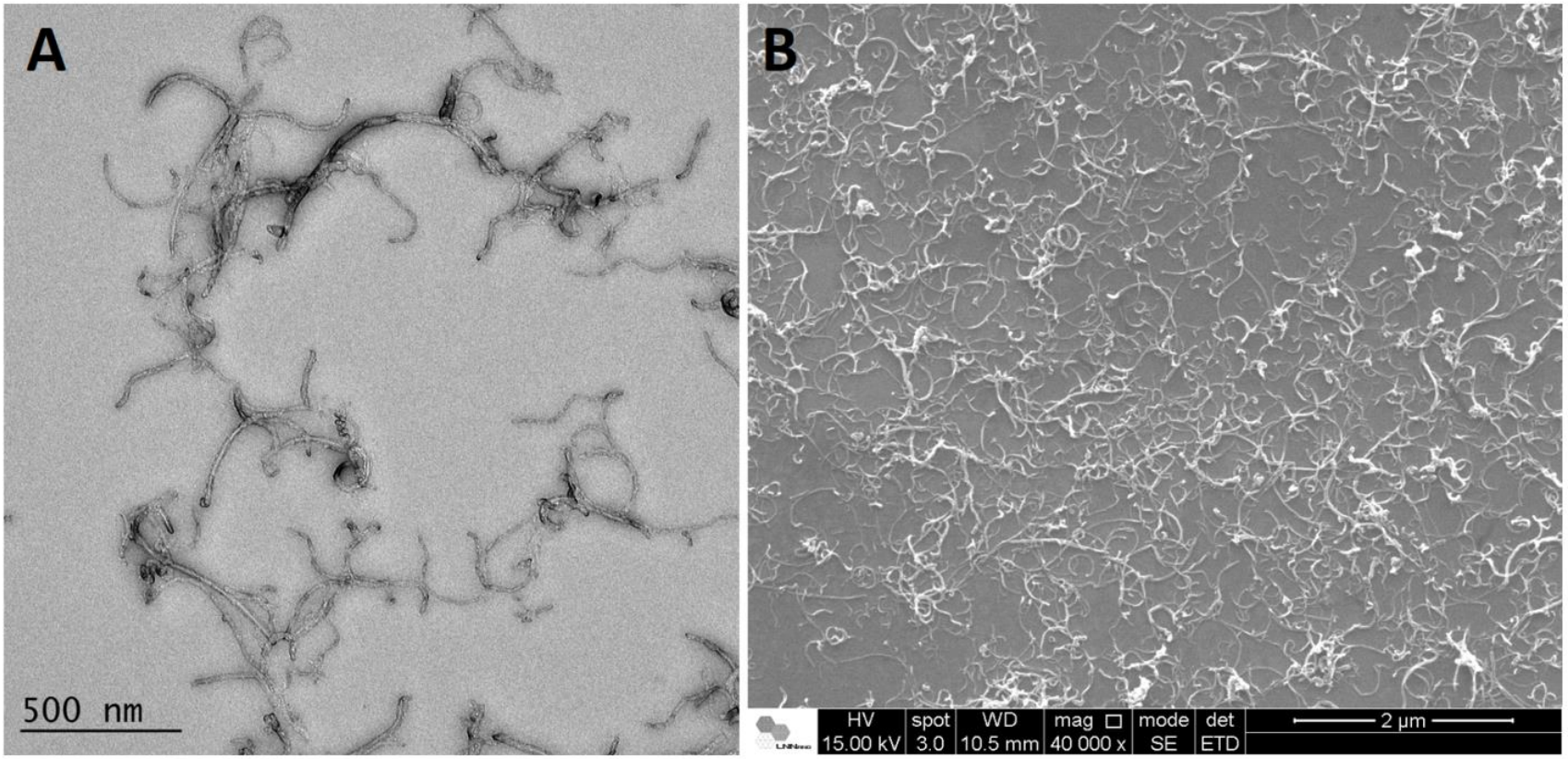

Figure 1. Morphology of oxidized multiwalled carbon nanotubes (ox-MWCNT). **A.** Transmission electronic microscopy (TEM); **B**. Scanning electronic microscopy (SEM).

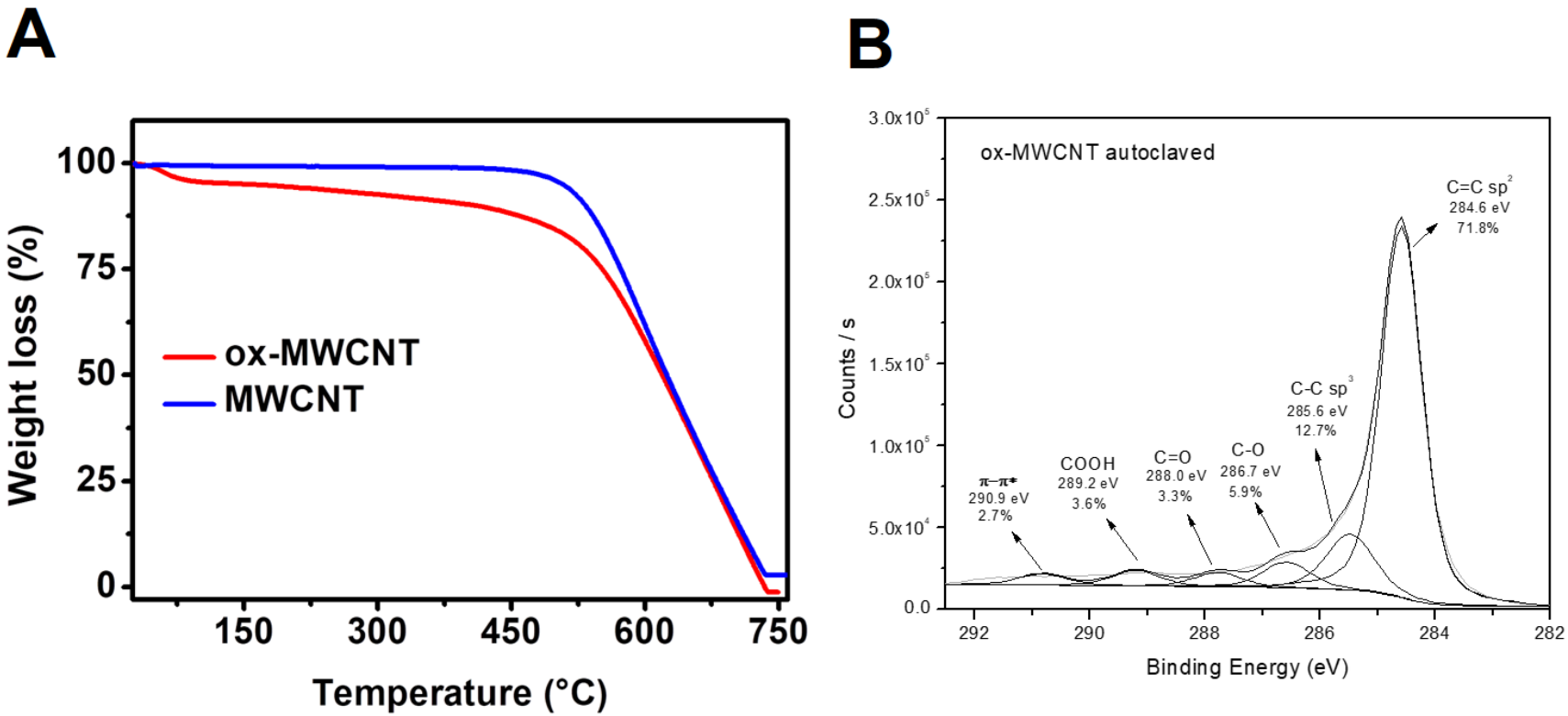

Figure 2. A. Thermogravimetric curve of bare multiwalled carbon nanotubes (MWCNT) and oxidized multiwalled carbon nanotubes (ox-MWCNT) at a heating rate of 5 ^{o}C min^{-1} from room temperature to 750 ^{o}C in a synthetic airflow of 50 mL min^{-1}. **B.** High resolution X-ray photoelectron spectrum of oxidized multiwalled carbon nanotubes (ox-MWCNT).

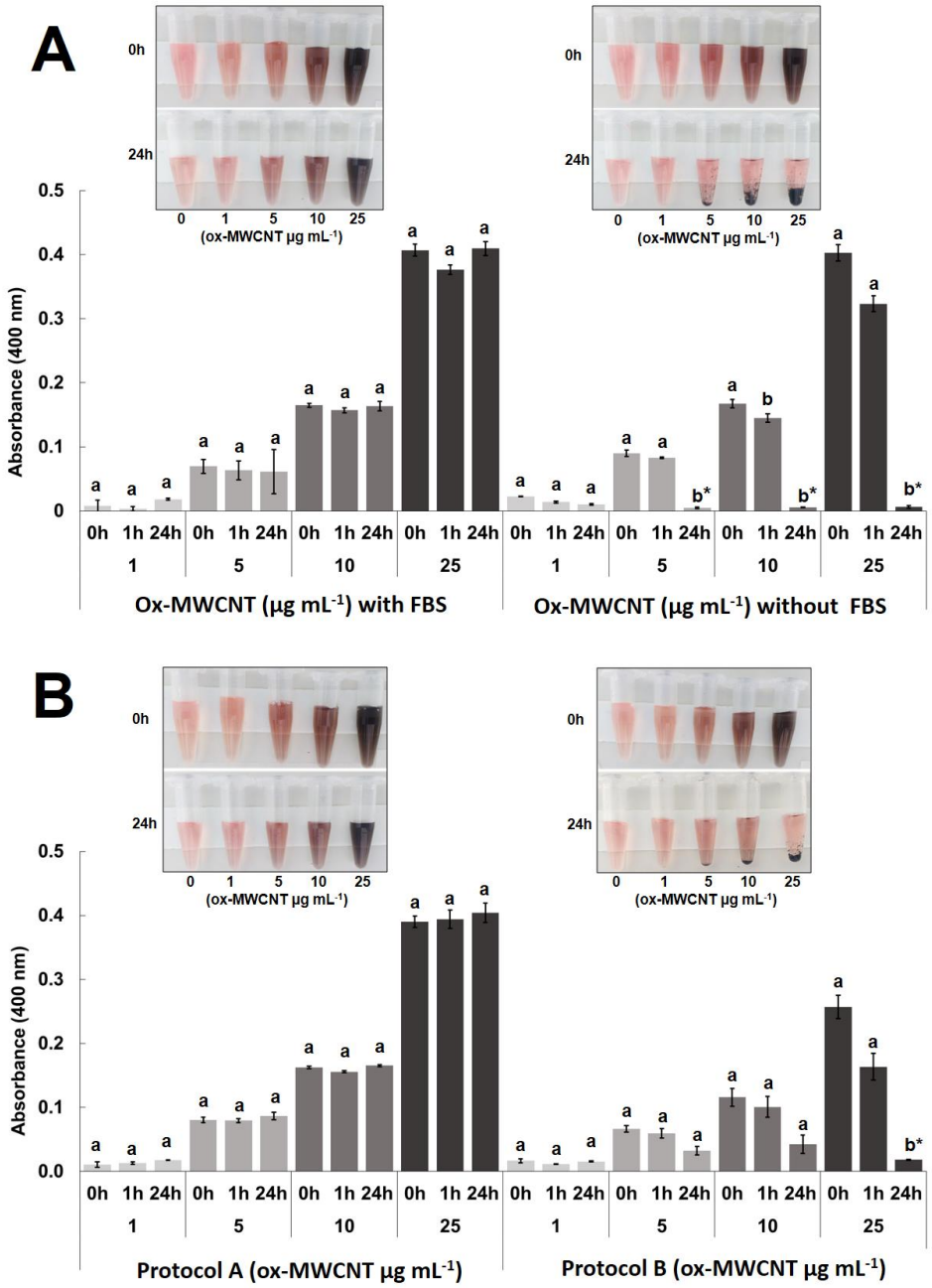

Figure 3. Dispersion and stability of 1, 5, 10 and 25 µg mL^{-1} of oxidized multiwalled carbon nanotubes (ox-MWCNT) for 24 hours using UV-Vis spectrophotometer analyses. **A.** Incubation of ox-MWCNTs in culture media (RPMI/L15) with and without addition of 10% fetal bovine serum (FBS); **B.** Protocol A, incubation of ox-MWCNTs in the culture medium (RPMI/L15) with FBS for 30 minutes and subsequent addition of 25 µg mL^{-1} of $CdCl_2$ for 30 minutes; thereafter, measurements were taken at 0, 1 and 24 hours. Protocol B, incubation of ox-MWCNT with 25 µg mL^{-1} of $CdCl_2$ for 30 minutes and subsequent addition of medium (RPMI/L15) with FBS for 30 minutes; thereafter, measurements were taken at 0, 1 and 24 hours. Different letters indicate differences over time for the same ox-MWCNT concentration. Asterisk indicates significant differences among the protocols for the same concentration (ANOVA, Tukey, $p<0.05$).

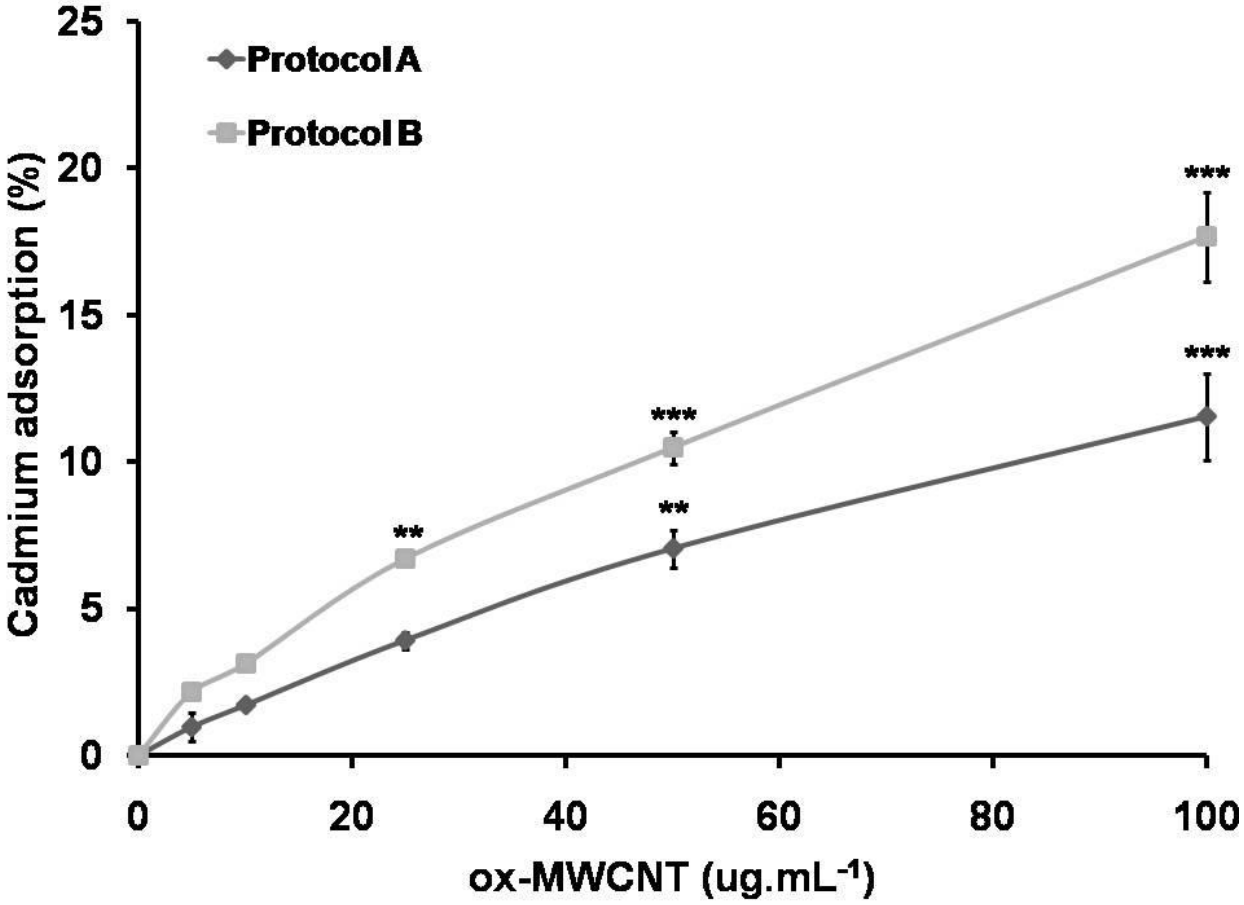

Figure 4. Cadmium adsorption by MWCNT in culture medium (RPMI/L15) supplemented with serum performed by ICP-OES. **Protocol A:** incubation protocol A followed by ultracentrifugation at 14,000 G (medium+FBS and MWNTC at 5, 10, 25, 50 and 100 µg.mL^{-1} for 30 minutes subsequent addition of $CdCl_2$ at 25 µg mL^{-1} for more 30 minutes); **Protocol B:** incubation protocol B followed by ultracentrifugation at 14,000 G (MWNTC at 5, 10, 25, 50 and 100 µg.mL^{-1} and $CdCl_2$ at 25 µg mL^{-1} for 30 minutes and subsequent addition of medium+FBS for more 30 minutes). Asterisks indicate differences between treatments and 0 µg mL^{-1} of MWCNT (Kruskal-Wallis test, ** $p<0.05$, *** $p<0.01$).

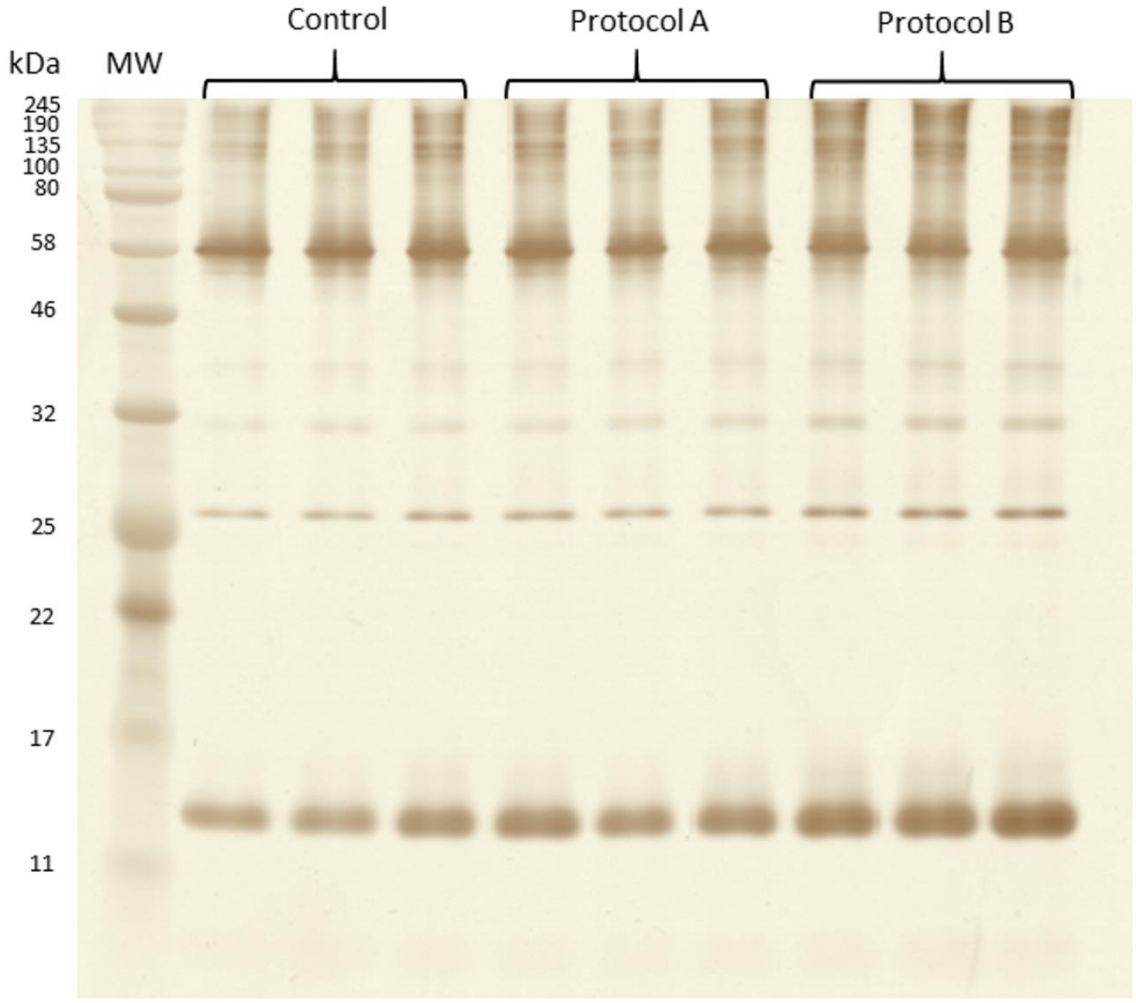

Figure 5. SDS-PAGE gel electrophoresis (15%) of fetal bovine serum (FBS) hard corona proteins extracted from ox-MWCNT obtained in protocols A and B (25 µg.mL^{-1} of ox-MWCNT and 25 µg.mL^{-1} of Cd) and ox-MWCNT (25 µg mL^{-1}) in the absence of Cd (control), after 1h incubation in RPMI/ L15 medium supplemented with 10% FBS.

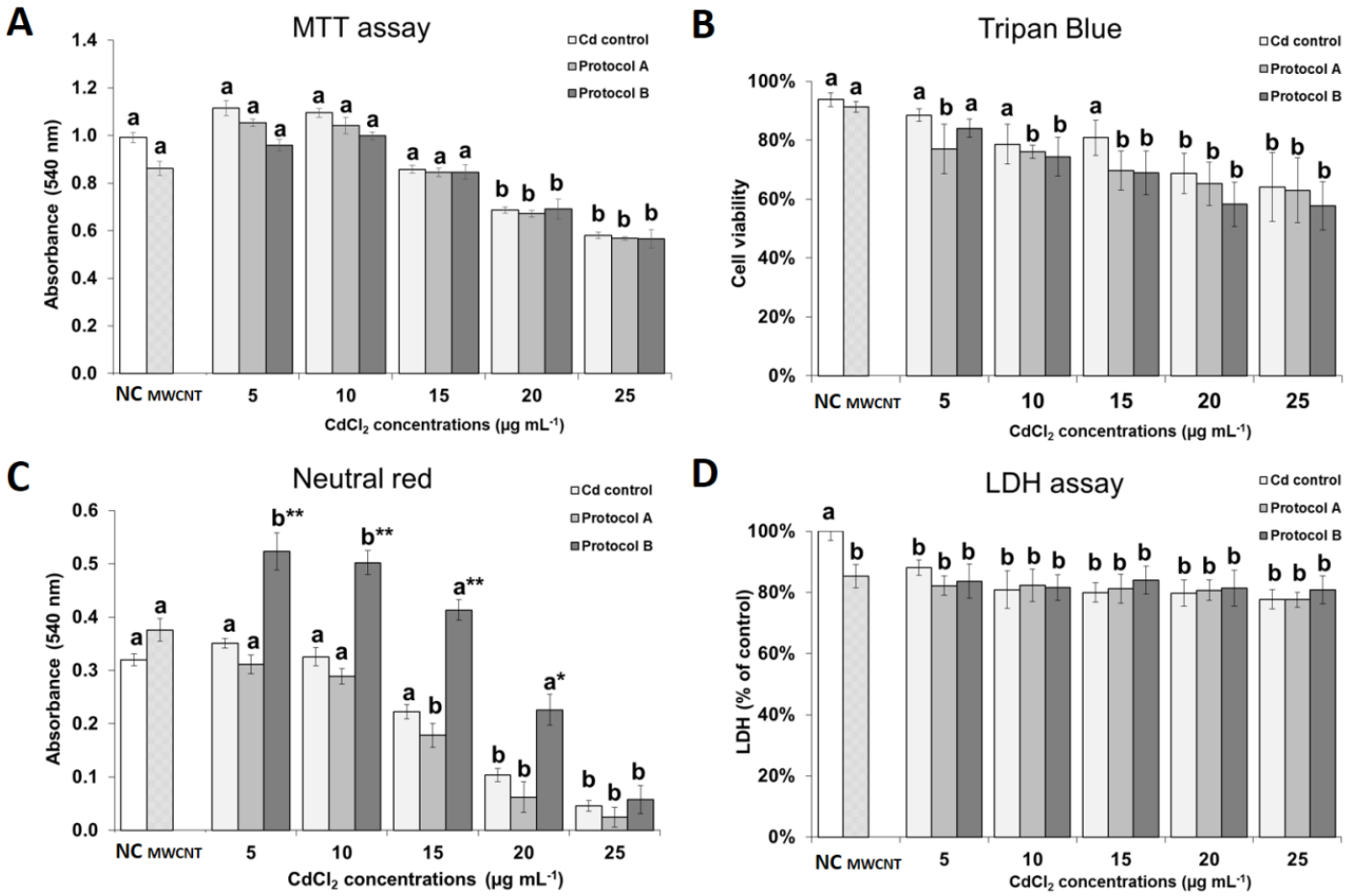

Figure 6. Cell viability results of ZFL cells exposed for 24 hours to different cadmium treatments and alternate exposure order of nanotube and RPMI/L15 medium supplemented with serum. **A.** Cell viability by MTT test; **B.** Cell viability by exclusion of trypan blue; **C**: Cell viability by neutral red; **D.** Cell viability by LDH assay. **NC**: negative control, RPMI/L15 medium supplemented with serum; **MWCNT**: oxidized multiwalled carbon nanotubes, RPMI/L15 medium supplemented with serum and 10 µg mL^{-1} of ox-MWCNTs. **Protocol A:** incubation of ox-MWCNTs in the culture medium (RPMI/L15) with FBS for 30 minutes and subsequent addition of $CdCl_2$ for 30 minutes. **Protocol B:** incubation of ox-MWCNT and $CdCl_2$ for 30 minutes and subsequent addition of medium (RPMI/L15) with FBS for 30 minutes. Different letters indicate significant differences from the negative control (ANOVA, Tukey, $p<0.05$). Asterisk indicates significant differences among the protocols for the same concentration (**) $p<0.05$, (*) $p<0.1$.

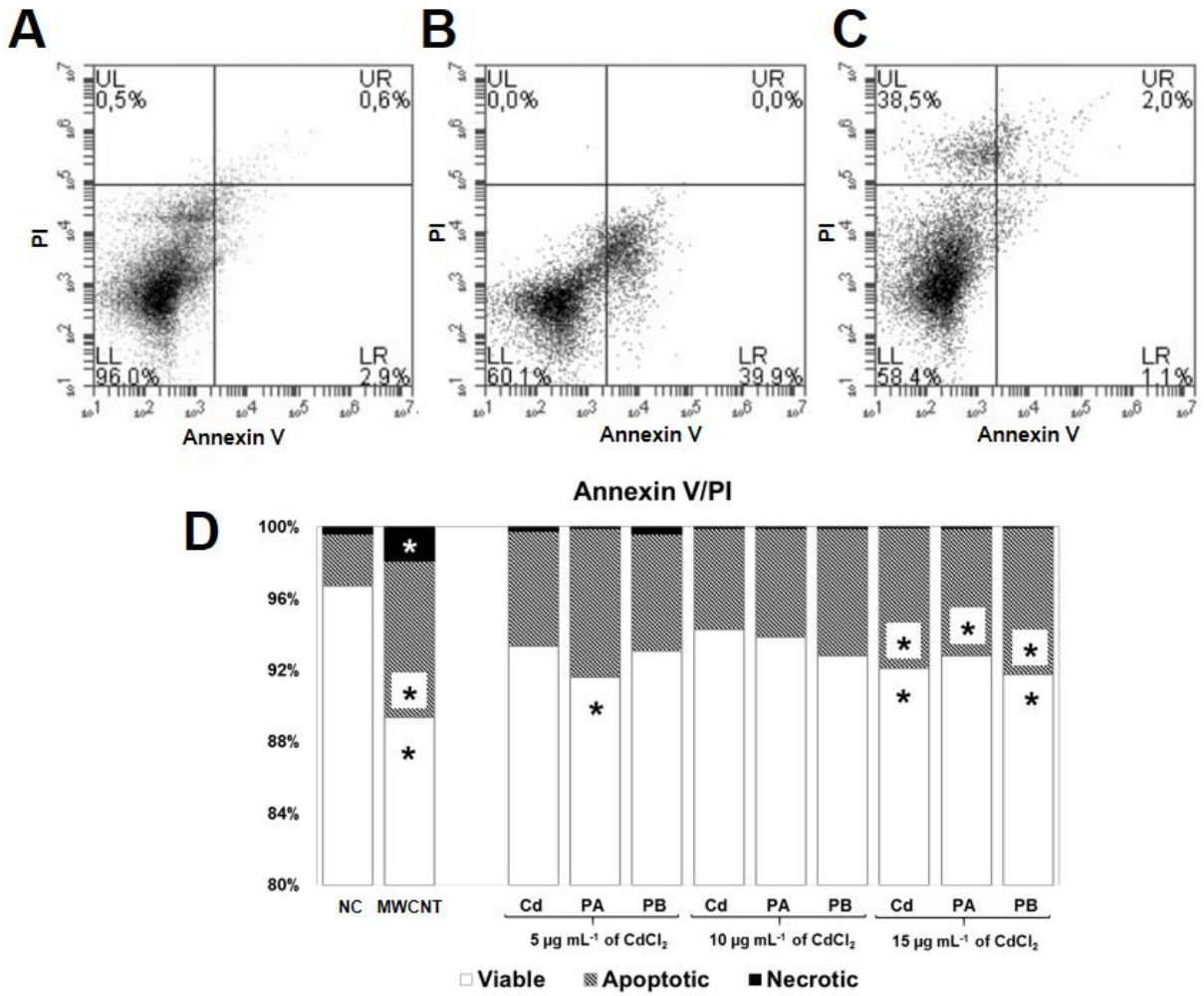

Figure 7. Flow cytometry analysis of Annexin-V-FITC and PI staining. ZFL cells exposed to different cadmium concentration and alternate exposure order of oxidized multiwalled carbon nanotubes (ox-MWCNT) and RPMI/L15 medium supplemented with fetal bovine serum, for 24 hours. **A.** Viable cells with double staining (Annexin-V and PI); **B.** Control Annexin-V staining; **C.** Control PI staining. LL: Annexin-V-/PI-cells (normal); LR: Annexin-V+/PI-cells (early apoptosis); UR: Annexin-V+/PI+ cells (late apoptosis); UL: Annexin-V-/PI+ cells (necrosis). **D.** Comparative data for other treatments. **NC:** negative control, RPMI/L15 medium supplemented with serum; **MWCNT:** oxidized multiwalled carbon nanotubes, RPMI/L15 medium supplemented with serum and (10 µg mL^{-1} of ox-MWCNT); **Cd:** treatments with medium supplemented with serum and $CdCl_2$; **PA:** treatments with protocol A, incubation of ox-MWCNTs in the culture medium (RPMI/L15) with FBS for 30 minutes and subsequent addition of $CdCl_2$ for 30 minutes; **PB:** treatments with protocol B, incubation of ox-MWCNT and $CdCl_2$ for 30 minutes and subsequent addition of medium (RPMI/L15) with FBS for 30 minutes. Asterisk (*) indicates significant differences (ANOVA, Tukey, $p<0.05$) compared to negative control.

Supplementary data

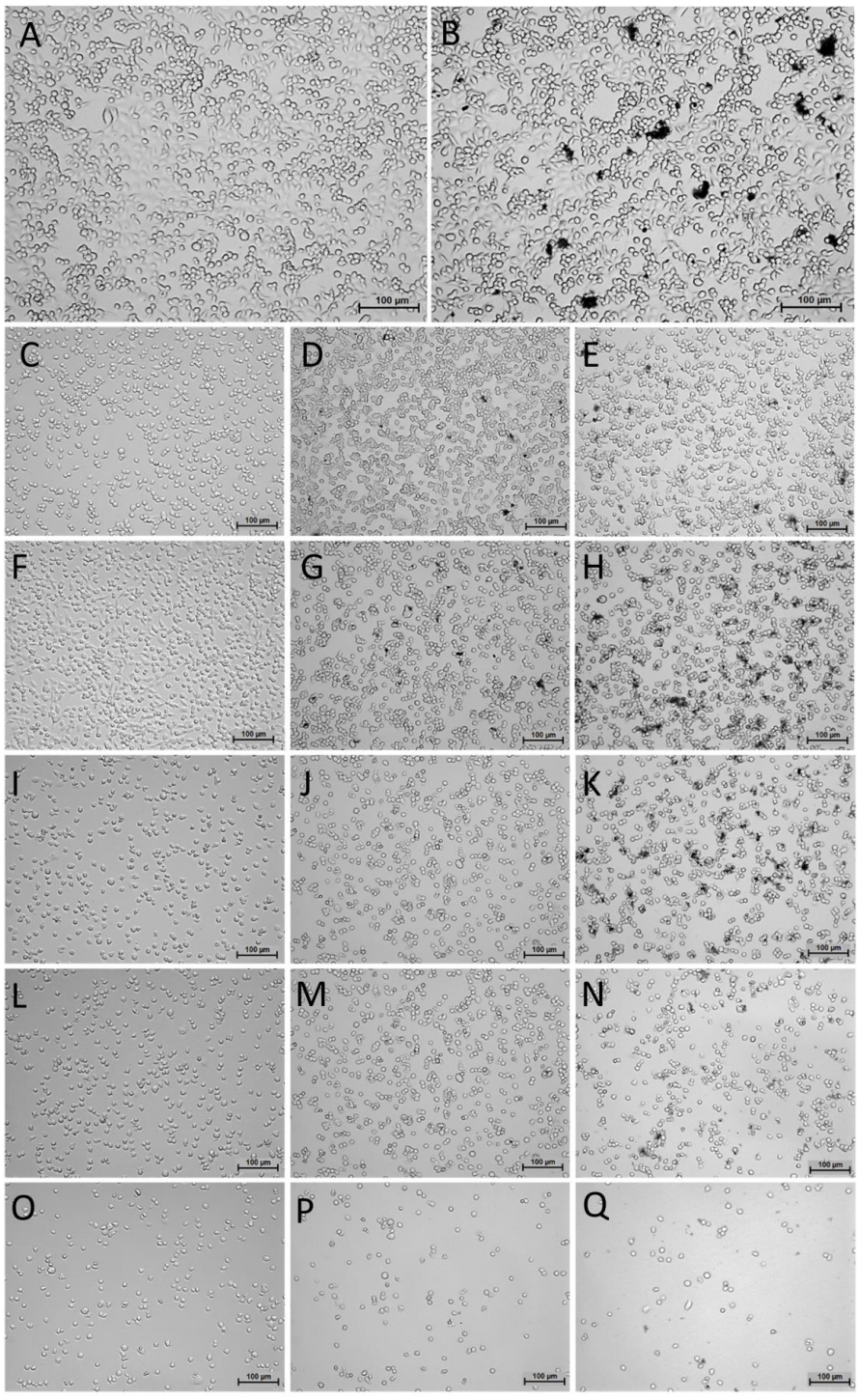

Figure S1. Confluence and morphology of ZFL cells by light microscope in the following treatments: **A:** negative control, RPMI/L15 medium supplemented with serum; **B**: ox-MWCNT (oxidized multiwalled carbon nanotubes) 10 µg mL^{-1}; **C, F, I, L and O**: cells exposed to 5, 10, 15, 20 and 25 µg mL^{-1} of $CdCl_2$; **D, G, J, M and P**: cells exposed to Protocol A, in order ox-MWCNT of 10 µg mL^{-1} and 5, 10, 15, 20 and 25 µg mL^{-1}; **E, H, K, N and Q**: cells exposed to Protocol B, in order to 5, 10, 15, 20 and 25 µg mL^{-1} of Cd and ox-MWCNT of 10 µg mL^{-1}

4.2 ARTIGO II

Após correções este artigo científico será submetido à revista **Archives of Toxicology**, ISSN 0340-5761 (impresso); 1432-0738 (online).

Modulation of cadmium cellular effects by multiwalled carbon nanotubes: quantifying metal uptake, oxidative stress and genotoxicity on zebrafish cell line

Abstract

Carbon nanotubes presence in the environment increases every year as a result of exponential industrial production around the world. In aquatic environments, carbon nanotubes can interact with other pollutants based on their nanoadsorbent surface chemistry properties. Heavy metal ions represent one of the biggest concerns in water resources nowadays, as a consequence of anthropogenic actions, in which cadmium (Cd) is one of the most harmful metal for aquatic organisms. Considering co-exposure nanoecotoxicity studies, this study investigated the influence of two incubation protocols of oxidized multiwalled carbon nanotubes (ox-MWCNT) with Cd on zebrafish liver cell line (ZFL). The co-exposure protocols differed by the order of ox-MWCNT interaction with Cd and fetal bovine serum (FBS) proteins in cell media. The ox-MWCNT studied was characterized, Cd content and uptake by cells were analyzed using ICP-MS and ten biochemical biomarkers [i.e. reactive oxygen species production (ROS), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), reduced glutathione (GSH), glutathione S-transferase (GST), lipid oxidation (TBars method), carbonylated proteins, and metallothionein content] were used to evaluate the effects of these interactions on ZFL. The effects on cell cycle were investigated by flow cytometry and DNA damage by comet assay. We demonstrated that ox-MWCNT in both protocols increases the ZFL content of cadmium after 24 hours of exposure and the order of incubation of each protocol used for ox-MWCNT and Cd interaction modified the nanotube-metal complexes formed. The treatment with only ox-MWCNT promotes depletion of catalase, glutathione peroxidase,

and glutathione S-transferase and led to alterations in cell cycle with a reduction of cells in G2/M phase. The two co-exposure protocols increase the cellular Cd effect in ROS production and DNA damage in ZFL cell line without differences between them. Finally, our study contributes to future co-exposure toxicity investigations and nanosafety regulations involving carbon nanomaterials and aquatic pollutants.

Keywords: MWCNT, ZFL, nanoecotoxicology, nanotoxicity, heavy metals and nanosafety.

1. Introduction

The unique nanometer-scale structure of carbon nanotubes (CNTs) associated with their physical and chemical properties allows its application in a wide range of industrial products (Cimbaluk et al., 2018; Bhanjana et al., 2017; Ghosh et al., 2015). Multiwalled carbon nanotubes (MWCNTs) usually consist of 2–50 graphene cylinders with few nanometers in diameter concentrically stacked with a common long axis (Öner et al., 2018; Siegrist et al., 2014). This nanomaterial is widely used and their presence in aquatic environments increases each year; into water they can interact with other pollutants due to their high adsorbent surface (Girardello et al., 2015; Campos-Garcia et al., 2015; Filho et al., 2014).

Metals ions represent one of the biggest concerns in water as a consequence of effluent releases, industrialization process and agricultural practices (Souza et al., 2013; Rao et al., 2007). Cadmium (Cd) is a toxic and ubiquitous element that promotes oxidative stress, alterations in cell cycle and DNA damage repair in fish (Guo et al., 2017; Olsvik et al., 2016). Cd is a primary pollutant in aquatic environments that proved to be lethal to water organisms at high concentrations; moreover this metal can bioaccumulate in the organisms and be biomagnified until higher trophic levels, such as humans (Zheng et al., 2016). Some studies suggests that liver is a primary target of Cd in these organisms than other organs (Balmuri et al., 2017; Yeşilbudak and Erdem, 2014; Chen et al., 2014).

Among the aquatic species, zebrafish (*Danio rerio*) is a model in environmental science due to complete genome sequence and a large number of comparative studies that facilitates to understand mechanisms in toxicology (Zheng et al., 2016; Eide et al., 2014). In this study, zebrafish liver cell line (ZFL) was selected to investigate the biochemical effects inside cells in response to exposure to oxidized MWCNT (ox-MWCNT) combined with Cd. ZFL cells show a general hepatocyte morphology characteristic that makes them useful in toxicological and detoxification investigations and allows an accurate interpretation of pollutants potential risks (Chen et al., 2014).

In *in vitro* systems, nanomaterials can interact with cell medium and it can change the composition of their surface through the contact with fetal bovine serum

proteins (Mahmoudi et al., 2016; Winzen et al., 2015). Carbon nanotubes adsorption properties can attract proteins of serum that cover all the nanomaterial structure forming a “protein corona”, which becomes the first contact with cells and may influence the interactions, accumulation and uptake mechanism of CNT (Walczyk et al., 2010; Zhao et al., 2015; Bhattacharya et al., 2016).

Despite the debate concerning about the toxicity of the nanomaterials, it is evident that their high reactivity properties may potentially affect biological systems by direct or indirect action or through interaction with other compounds (Ferreira et al., 2014; Naasz et al., 2018). Thus, what happens when nanomaterials interact with other toxic pollutants?

Due to the large surface area and many oxygenated surface chemistry groups (negatively charged), ox-MWCNT act as an adsorbent of Cd and others metals and reduce their levels into the environment (Bhanjana et al., 2017; Rao et al., 2007). Conversely, they act as a concentrator and contaminant carrier to organisms enhancing their bioavailability and toxicity, as a “trojan horse effect” (Deng et al. 2017; Ferreira et al., 2014; Martinez et al., 2013). However, few studies investigate the interactions of these two types of pollutants and the toxicity mechanisms and cellular effects remain scarce currently (Morozesk et al., 2018; Naasz et al., 2018; Wang et al., 2016).

In the previous study, the influence of interaction order of ox-MWCNT and Cd in co-exposure toxicity studies were demonstrated emphasizing that the order of compounds interactions disturbs the ox-MWCNT colloidal stability, affects its adsorption capacity and effects on cell viability (Morozesk et al., 2018). Now, we aimed to demonstrate the ox-MWCNT modulation in cell uptake of cadmium as well as biochemical and genotoxicity effects of these combined pollutants on zebrafish liver cell line.

2. Materials and Methods

2.1. Preparation and characterization of oxidized multiwalled carbon nanotubes

The nitric acid-oxidized multiwalled carbon nanotube (ox-MWCNT) used in this work was prepared and characterized according to Morozesk and collaborators (2018). Briefly, ox-MWCNT presented a weight loss about 8% at temperatures below 500°C (Thermogravimetric analysis - TGA, 449 F3 Jupiter@, NETZSCH) due to the decomposition of oxygenated functional groups on its surface and, a surface charge of −62.8 ± 2.15 mV (ζ-potential, (Zetasizer Nano-Instrument, Malvern). The oxygen content was about 6.89 % with a total oxidation degree (DOxid) of 0.15 (K-Alpha photoelectron spectrometer - XPS system, Thermo Fisher Scientific). To biological studies, the ox-MWCNT was previously autoclaved and a stock-dispersion was prepared at 0.5 mg mL^{-1} in ultrapure water by sonication for 60 min (Ultrasonic Bath, Cole-Parmer 08895-43). The morphology and structure of ox-MWCNT was preserved after sterilization process (Transmission Electron Microscopy – TEM, JEM-1400 plus, JEOL).

2.2. Incubation protocols of ox-MWCNT with Cd to zebrafish liver cell co-exposure treatments

In this study, the following treatments were performed: (I) negative control, with only buffer solution (PBS), (II) ox-MWCNTs (5 µg mL^{-1} and 10 µg mL^{-1}), (III) cadmium controls (1, 5, 10 µg mL^{-1} of $CdCl_2$), (IV) incubation protocol A (medium+FBS with 5 µg mL^{-1} of ox-MWCNTs for 30 minutes and subsequent addition of 1, 5, 10 µg mL^{-1} of $CdCl_2$ for more 30 minutes), (V) incubation Protocol B (5 µg mL^{-1} of ox-MWCNT with 1, 5, 10 µg mL^{-1} of $CdCl_2$ for 30 minutes and subsequent addition of medium+FBS for more 30 minutes). Positive controls with methyl methanesulfonate (MMS, 0.5 mM for 24h) and hydrogen peroxide (Pex, 50 µM for 30 min) was performed in ROS and comet assay to validate the protocols. Hydrogen peroxide was chosen based in their capacity to induce oxidative stress damage and MMS is an alkylating agent that promotes strand breaks in DNA (Cavalcante et al., 2014). After these procedures, the treatments were applied in ZFL cells in *in vitro* conditions for 24h.

2.3. Culture conditions

Liver cells from zebrafish, ZFL (*Danio rerio*) was provided by Rio de Janeiro Cell Bank, Brazil, and were cultivated (passage numbers 15–25) in RPMI/L-15 culture medium (Cultilab, Brazil), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Plates were maintained in a BOD type incubator at 28 ºC in air atmosphere 100%. Thereafter, cells were transposed to 75 cm^2 flasks using $9x10^6$ cell/flask concentration for cadmium uptake determination and biochemical analysis, to 96-well plates at $2.5x10^5$ cell/well concentration for ROS analysis and to 6-well plates at $8x10^5$ cell/well concentration for cell cycle analysis.

2.4. Determination of cadmium uptake

The Cd analysis was performed using an Agilent 7800 Quadrupole ICP-MS (Agilent Technologies, Tokyo, JHS, Japan) and argon (99.9996%, White Martins-Praxair, Sertãozinho, SP, Brazil) was used for all measurements. HNO_3 (Merck, Kenilworth, NJ, Brazil) purified in a sub-boiling distillation system DistillacidTM BSB-939-IR (Berghof, Eningen, Germany) and ultrapure water, resistivity higher than 18.2 MΩ cm, (Milli-Q®, Millipore, Bedford, MA, USA) were used for organic material digestion.

After treatments, the medium was reserved and ZFL cells was washed with PBS three times, detached from flasks using trypsin (0.25%), centrifuged at 2000 rpm for 5 minutes and dried at 60 ºC until reach a constant weight. Aliquots of 1 mL of reserved medium, before and after the treatments, and 0.005 g of dried cells were digested separated (triplicate) in digester units using 5 mL of distilled HNO_3 at 100º C for 24 h in a ceramic plate. Subsequently, the digested content was filtered in 45 µm PFTE membrane (polytetrafluoroethylene), weighted and diluted up to twenty times with ultrapure water for analysis. Controls of method were prepared, in triplicate, using the same procedure without sample (only reagents). Standard solutions used for ICP-MS calibration, for addition and recovery experiments were prepared by diluting 1000 mg L^{-1} Cd (Qhemis, São Paulo, Brazil) in 0.14 mol L^{-1} HNO_3 medium. The concentrations of solutions to obtain the calibration curves were 0, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 12, 16, 20

and 50 µg L^{-1}. The accuracy of the ICP-MS analytical procedure was evaluated by addition and recovery experiments and by analysis of the certified reference material bovine liver (NIST SRM 1577b). The certified reference material of bovine liver NIST SRM 1577b (National Institute of Standard and Technology, Gaithersburg, MD, EUA) was used to evaluate accuracy, addition and recovery experiments and was performed at three addition levels (2, 4 and 8 µg L^{-1}). The detection limits (LOD) was calculated considering standard deviation (SD) for 10 measurements of a blank solution (Thomsen et al., 2003). The isotopes $^{111}Cd^{+}$, $^{112}Cd^{+}$ and $^{114}Cd^{+}$ were monitored and reported as the $^{112}Cd^{+}$ based on LOD and linearity of analytical calibration curve. The recovery and relative standard deviation obtained for Cd was 93±5%, being that the certified value 0.5±0.03 µg g^{-1} and the determined value 0.465±0.025 µg g^{-1}, and suitable recoveries, i,e, 97±3% was also obtained in addition and recovery experiments. The LOD, linearity of analytical calibration curve and sensitivity for ICP-MS analysis were 0.0014 µg L^{-1}, 39215 counts L/s μg^{-1} and 0.9997, respectively.

2.5. Biochemical analysis

ROS determination was performed according to Cavalcante et al. (2014) and Zhu and Chan (2012) with adaptations. After the exposure periods, the cells were washed two times with PBS; medium (RPMI/L-15) without serum with 25 µM of 2,7 dichlorofluorescein diacetate (H_2DCFDA) was added and cells were incubated in dark for 30 minutes. Then, the cells were washed with PBS, trypsinized and resuspended in medium supplemented with serum. After that, the cells were transferred to dark well plate, centrifuged at 1500 rpm for 5 min and resuspended in PBS. The spontaneous fluorescence of each sample at excitation and emission wavelengths of 488 and 525 nm, respectively, was measured in a spectrofluorometer programmed to operate at 28 ºC.

For other biochemical analysis, cell homogenates were prepared according Cavalcante et al. (2014) with sonic dismembrator, model 500 (Fisher Scientific, USA). Total protein content of each sample was determined according to the Bradford method (1976) at 595 nm using bovine serum albumin as standard and nine biochemical

biomarkers were evaluated. The enzyme activities were determined as following: superoxide dismutase (SOD) by the inhibition of the reduction rate of cytochrome c by the superoxide radical as described by McCord and Fridovich (1969); catalase (CAT), by monitoring the H_2O_2 decomposition rate as described by Beutler (1975) and Aebi (1984),; glutathione peroxidase (GPX) by consumption of $NADPH^+H^+$ in the presence of peroxide as described by Beutler (1975) and Gallagher *et al.* (1992); glutathione reductase (GR) as described by Nagalakshmi and Prasad (2001) by reduction of glutathione disulfite (GSSG) by oxidation of $NADPH^+H^+$; and the biotransformation enzyme glutathione S-transferase GST, as described by Habig and Jakoby (1981) by 1-chloro-2,4-dinitrobenzene (CDNB) reaction with GSH.

Moreover, the reduced glutathione content (GSH) was determined by naphthalene-2,3-dicarboxaldehyde (NDA) reaction as described by White *et al.* (2003) and metallothionein content by Viarengo *et al.* (1997) using Ellman's Reagent (5,5'-dithio-bis-[2-nitrobenzoic acid]). Lipid peroxidation was determined according to Oakes and Van Der Kraak (2003) using thiobarbituric acid reactive substances (TBars method) and carbonylated proteins were quantified by Levine *et al.* (1994) using 2, 4-dinitrophenylhydazine (DNPH) and all measurements were adapted for a microplate reader (SpectraMax M5, Molecular Devices, USA).

2.6. Cell Cycle

After the exposure periods, the cells were detached, centrifuged and washed with cold PBS, fixed in 70% cold ethanol and stored at -20 °C for 24 h. Fixed cells were incubated with RNase A (0.2 mg ml^{-1}) (Sigma, St. Louis, MO, USA) at 37 °C for 30 min and stained with iodide propidium (PI, 1 µg ml^{-1}) (Sigma, St. Louis, MO, USA) according Martin *et al.* (2017). DNA content in cells was measured in 20,000 events with flow cytometer Accuri C6 (BD Bioscience, NJ, USA).

2.7. Alkaline comet assay

DNA damage in cells exposed to each treatment described before were analyzed by the alkaline comet assay, which detects only DNA strand breaks and alkali-labile sites. At the end of exposure time, cell medium was removed and the flasks were washed with PBS, the cells were trypsinized and resuspended in medium with FBS. Then, the cells were centrifuged and the supernatant was discarded, the pellet was used for the preparation of slides for the comet assay, following the protocol described by Singh *et al.* (1988) with slight modifications (Cavalcante et al., 2014). All analysis was performed in a fluorescence microscope under 40x objective lens magnification, using a 510–560 nm excitation filter and a 590 nm barrier filter. Six hundreds nucleoids were analyzed per treatment using the OpenComet software (Gyori et al., 2014) and values of comets area, olive tail moment (OTM) and % of DNA in tail provided by software analysis were reported. OTM was calculated using the percentage of DNA in the tail and the distance between the intensity centroids (centers of gravity) along the comet x-axis (Kurumavel et al., 2009).

2.8. Statistical analysis

Shapiro-wilk normality test was applied in all data to check normality following by ANOVA and Tukey's test ($p < 0.05$) for parametric data or Kruskal-Wallis test ($p < 0.05$) for nonparametric data using Infostat software (Di Renzo et al., 2010). Experimental conditions were performed in 3-4 biological replicates and 3-5 technical replicates.

3. Results

3.1. Characterization of ox-MWCNT

Figure 1 shows the morphology of ox-MWCNT, hydrodynamic particle size and zeta potential of ox-MWCNT and final complexes after interaction with $CdCl_2$ in Protocols A and B. The size of complexes formed in Protocol B was significantly higher than those formed in Protocol A (Fig. 1A). Zeta potential of ox-MWCNT increased from -

62.8 ± 2.15 mV to -32.0 ± 1.13 mV after interaction with cadmium in protocol A and to -32.5 ± 0.71 mV in Protocol B (Fig. 1D).

3.2. Cadmium uptake

The content of Cd in the initial and final medium and inside the cells in each treatment was presented in Figure 2. As expected, the increasing $CdCl_2$ concentration in media at initial and final exposure period in each treatment was followed by increasing cadmium content inside cells. No differences occurred in the medium at initial and final exposure period between the protocols for the same $CdCl_2$ concentration. However, cadmium content inside cells was higher in Protocol A compared to control Cd at 10 μg mL^{-1}. Figures 2D, 2E, and 2F show cell confluence in negative control and in Protocol A and B, respectively; in which it is possible observed ox-MWCNT agglomerates in the medium in Protocol B.

3.3. Biochemical biomarkers

ROS production in negative control differed from all treatments at 5 and 10 μg mL^{-1} of $CdCl_2$ and from positive controls MMS and hydrogen peroxide. The results of Protocols A and B performed within the same cadmium concentration showed significant differences between Cd control and protocol A at 10 μg mL^{-1} (Fig. 3).

Superoxide dismutase activity (SOD) was reduced in Protocol A at 1 μg mL^{-1} and Cd at 10 μg mL^{-1} of $CdCl_2$ in comparison to negative control. Differences were observed between Protocols A and B at 10 μg mL^{-1} of $CdCl_2$ (Fig. 4A). Catalase activity (CAT) was reduced in all protocols and treatments with $CdCl_2$ including ox-MWCNT only and differences among Protocol A and B at 10 μg mL^{-1} were observed (Fig. 4B). Glutathione peroxidase activity (GPX) decreased in ox-MWCNT treatment and 1 and 5 μg mL^{-1} of $CdCl_2$ treatments of Protocol B (Fig. 4C). Glutathione reductase activity (GR) and reduced glutathione content (GSH), in all treatments, did not differ from negative control (Fig. 4D and F). The activity of biotransformation enzyme glutathione S-transferase (GST) was reduced in ox-MWCNT treatment, in all treatments at 1 μg mL^{-1} of $CdCl_2$ and

in Cd exposure at 5 µg mL-1 (Fig. 4E). Metallothionein content presented higher values in all treatments compared with negative control. Differences among Cd treatment and protocol A and B at 1 µg mL^{-1}, Cd treatment and protocol A and B at 10 µg mL^{-1} were observed (Fig. 4G) in which cadmium treatment presented higher values than both protocols. Products of lipid peroxidation were higher in all treatments, except for ox-MWCNT (Fig. 5A). No differences were observed in carbonylated proteins content between treatments and negative control (Fig. 5B).

3.4. Cell cycle and genotoxicity

In this study, increases in sub G1 cells population and reduction of cells in phase G2 and mitosis were observed in all $CdCl_2$ treatments compared with negative control. Cell population in phase G0 and G1 was reduced at all concentrations of protocol B and at 10 µg mL^{-1} in protocol A and Cd control. Ox-MWCNT treatment also reduced the cells population in phase G2 and mitosis and enhanced cells in S phase at 5 µg mL^{-1} (Fig. 6).

Genotoxicity, measured as a percentage of DNA content in comet tail, showed alterations in all treatments except for ox-MWCNT. Differences among Cd treatment and protocol B were observed at 10 µg mL^{-1} of $CdCl_2$ (Fig. 7). In order to compare data of comet assay and cell cycle, the area and olive tail moment (OTM) of comets evaluated were presented in Table 1. Comet area was higher in negative control than $CdCl_2$ treatments at 10 µg mL^{-1} from Protocols A and B. Additionally, OTM presented the same pattern result than % of DNA in tail (Fig. 7).

4. Discussion

In a recent study of our group, we showed that ox-MWCNT partially loses its stability when first interact with $CdCl_2$ (Protocol B) (Morozesk et al., 2018) by the formation of aggregates which explain high size values of this treatment when compared with ox-MWCNT and protocol A (Fig. 1C). As demonstrated in previous

study, zeta potential values refers to the charge of proteins on nanotube surface, supported by similar values found by both complexes (Protocol A and B) in Figure 1D.

Oxidized carbon nanotubes can change the biodisponibility of metal ions by adsorption of them in its surface due to the presence of negatively charged oxygenated groups (Perez-Aguilar et al., 2011; Martinez et al., 2013). Cadmium cell content evidenced dose dependence in Cd absorption (Fig. 2). The higher intracellular Cd values in the Protocols A at the same Cd concentration alone demonstrate that the presence of ox-MWCNT enhance the uptake of Cd by ZFL cells (Fig. 2C).

Chen *et al.* (2014) already demonstrated that the uptake of Cd in ZFL cells is dose and time dependent. The strong affinity of CNTs for ionic and organic species, hydrophobicity, hollow structure and large surface area makes this nanomaterial an efficient sorbent for the removal of heavy metal ions and organic contaminants (Sengupta and Gupta, 2017). The adsorption capacity of Cd^{2+} ions by MWCNT was demonstrated by several studies (Rao et al., 2007; Perez-Aguilar et al., 2011; Bhanjana et al., 2017; Morozesk et al., 2018); however, it also favors cell uptake. The Cd content in ZFL in Protocols A and B, at 10 μg mL^{-1}, were related to its capacity of facilitating the entrance of contaminants by adsorption mechanism as a “Trojan horse” (Deng et al. 2017, Campos-Garcia, 2015, Ferreira et al. 2014).

Many mechanisms acting in adsorption of metal ions onto CNTs, some of them are related to electrostatic attraction, sorption–precipitation and chemical interaction between the metal ions and the surface functional groups of CNTs (Rao et al., 2007). Perez-Aguilar *et al.* (2011) showed that the formation of aggregates of carbon nanotubes can also influences the adsorption process by pore volume diffusion through the space between nanotubes. Thus, the order of interaction and the presence of high size and aggregates in protocol B might act by facilitating the Cd deposition above cells affecting its uptake on cells. Aggregates can be observed above the cells in Figure 2F of protocol B, compared with Protocol A and Cd control at 10 μg mL^{-1} in Figure 2D and E.

The mechanism of Cd induced carcinogenesis is still open for discussion, some proposal are related to disruption of the cellular antioxidant system and induction of reactive oxygen species (Cuypers et al., 2018; Guo et al., 2017; Liu et al., 2009),

disruption in cellular signal transduction (Thevenod, 2009), promotion of cell proliferation (Templeton and Liu, 2010) and inhibition of DNA repair (Hsu et al., 2013; Pierron et al., 2014). MWCNT had also promote high levels of intracellular reactive oxygen species (ROS) in macrophages (Di Giorgio et al., 2011), human fibroblast lung cells (BEAS-2B and WI38-VA13) (He et al., 2011), human adrenocortical carcinoma cells (T47Dluc), human adrenocarcinoma cells (H295R) and rainbow trout liver cells (RTL-W1) (Simon et al., 2014). However, in our study ox-MWCNT treatment only do not induced ROS production (Fig. 3) probably due to low concentration tested (5 μg mL^{-1}) or differences in the properties of ox-MWCNT in the mentioned studies in the literature (eg. groups on surface, size and characteristics of dispersion and stability). Therefore, the ROS production observed suggested synergic effects of Cd and ox-MWCNT at 10 μg mL^{-1}, in which the presence of ox-MWCNT enhances the ROS production in both protocols compared to Cd results.

The treatment with just ox-MWCNT affects catalase (CAT) and glutathione S-transferase (GST) enzymes by suppression of their activities. Several studies reported that MWCNT promote depletion of antioxidants enzymes, by reduction of gene expression and/or content, mediated by prooxidant effects (Lee et al., 2015, Simon et al., 2014). Lee *et al.* (2016) reported that enzymatic activity of catalase and glutathione S-transferase (GST) were significantly down-regulated in response to 100 mg L^{-1} of MWCNT exposure indicating a failure of the antioxidant system in a copepod specie (*Tigriopus japonicus*). Additionally, MWCNTs exposure promotes hypermethylation and downregulation of glutathione S-transferase pi 1 (GTSP1) gene in human bronchial epithelial cells (16HBE14o) (Öner et al., 2018).

The interaction of ox-MWCNT and $CdCl_2$ also promoted a decreasing activity of superoxide dismutase, catalase and glutathione S-transferase (Fig. 4) in some treatments. These results suggested that the combined effects of Cd toxicity and ox-MWCNT disturbed cell redox state, which may contribute to peroxide and free radical production and result in adverse effects on proteins, lipid peroxidation and DNA damage (Fu et al., 2014).

Emerging data suggest that metallothioneins have a close relationship with tumors; some of them have shown an increased expression of metallothioneins in liver

cancers (Balmuri et al., 2017; Cherian et al., 2003; Barnes et al., 2000). Ma *et al.* (2008) reported that Cd exposure clearly resulted in metallothioneins induction however, no correlation was observed between metallothionein synthesis and Cd accumulation at high concentrations of Cd exposure. In our data, the content of metallothionein proteins was higher in treatments without ox-MWCNT, which may suggest some interference of this nanomaterial in metallothionein detoxification action or influences in metallothionein genes expression.

To investigate the impact of alterations in the antioxidant system (enzymatic and non-enzymatic) and the biotransformation enzyme of phase 2, the content of lipid peroxidation and protein carbonylation were analyzed (Fig. 5). Lipid peroxidation is a major consequence of Cd-induced oxidative stress and metallothionein is a metal binding protein that has the role of protecting cells from Cd toxicity through Cd sequestration (Liu et al., 2009). Shvedova *et al.* (2003) reported that incubation of high doses of CNTs in bronchial epithelial cells produced ROS and lipid peroxidation. The increased ROS production and suppressed antioxidant enzyme activities led to enhanced of products of lipid peroxidation in co-exposure treatments with $CdCl_2$ and ox-MWCNT. The decreasing in the antioxidant defense system, the free radicals attack cell and sub-cellular components, including DNA, leading to oxidative damage. These damages on cell structures are characterized as irreparable and usually cause irreversible lesions in DNA structure and cell function (Gao et al., 2018).

Cell cycle regulation is crucial for the cell to maintain normal rate of proliferation in which alterations can lead to increased risk of cancer initiation (Öner et al., 2018). Cytotoxic activity can promote anti-proliferative effects with accumulation of cells in sub-G1 phase (Martin et al., 2017). Our data demonstrated that cadmium modifies the ZFL cell cycle leading to higher proportion of cells in sub-G1 phase (Fig. 6). In DNA content measured by comet assay, minor comets were observed in treatments with cadmium as a consequence of the lower number of cells populations in advanced phases of cell division cycle (Table 1). This is the first study that analyzed oxidized MWCNT effects in cell cycle of ZFL cells. The reduction of cells in G2/M phase and increase in S phase at 5 μg mL^{-1} promoted by ox-MWCNT, indicate a G1/S block and a failure to progress to

G2 and a similar found was reported by a previous study with human epithelial cells (Siegrist et al., 2014).

Due to the heterogeneity within a cell population, we also presented in Table 1 the parameter calculated by Olive *et al.* (2005) the Olive tail moment (OTM). This calculation is useful to describe cells in different phases of cell cycle and have good correlation with the dose of genotoxic agents used, been along with the parameter "% Tail DNA" one of the most reliable comet measurements (Kurumavel et al., 2009). OTM is in accordance with results showed in Figure 7 in which the increasing concentration of $CdCl_2$ promotes more DNA damage than the negative control. In OTM analysis, ox-MWCNT also presents damages in DNA in comparison to negative control which is not observed in % DNA in the tail.

All in all, our hypothesis for interactions of ox-MWCNT and Cd in this article is that Cd is the main factor to promote damage in cells by generation of reactive oxygen species. Ox-MWCNT can suppress CAT, GPX and GST in ZFL cells, acting on the biochemical line cell defense allowing the metal action and cell damage. Both contaminants can provoke alterations on cell cycle and the co-exposure protocols can promotes more damage than separate exposures by synergic effect. In our study, genotoxicity results are quite related with cadmium treatment concentration dependence and suggesting that the main mechanism of ox-MWCNT act to modulate Cd effects is by their influence in Cd cell uptake and antioxidant enzymes block, which increase the DNA damages in ZFL cells.

Conclusion

Co-exposure protocols of ox-MWCNT and Cd *in vitro* showed that the presence of FBS serum in the culture medium can affect the uptake of this metal on cells and enhance the genotoxic effects through the formation of different MWCNT-metal complexes. Treatment with only ox-MWCNT promotes the reduction of catalase, glutathione peroxidase, and glutathione S-transferase activities and changes cell cycle with reduction of cells in G2/M phase, which indicate a G1/S block. Although the different characterizations observed in Protocol A and B, the two co-exposure

treatments performed exhibited a similar pattern response in most of the biomarkers tested. However, it is important to understand the influence of the joint toxicity of Cd and ox-MWCNT as well as the differences and similarities about the two employed exposure protocols aiming to improve the nanoecotoxicity studies, selecting biomarkers and producing standardized and reproducible data using cell lines as a biological model.

Conflict of interest statement

The authors declare no conflict of interest.

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Figures

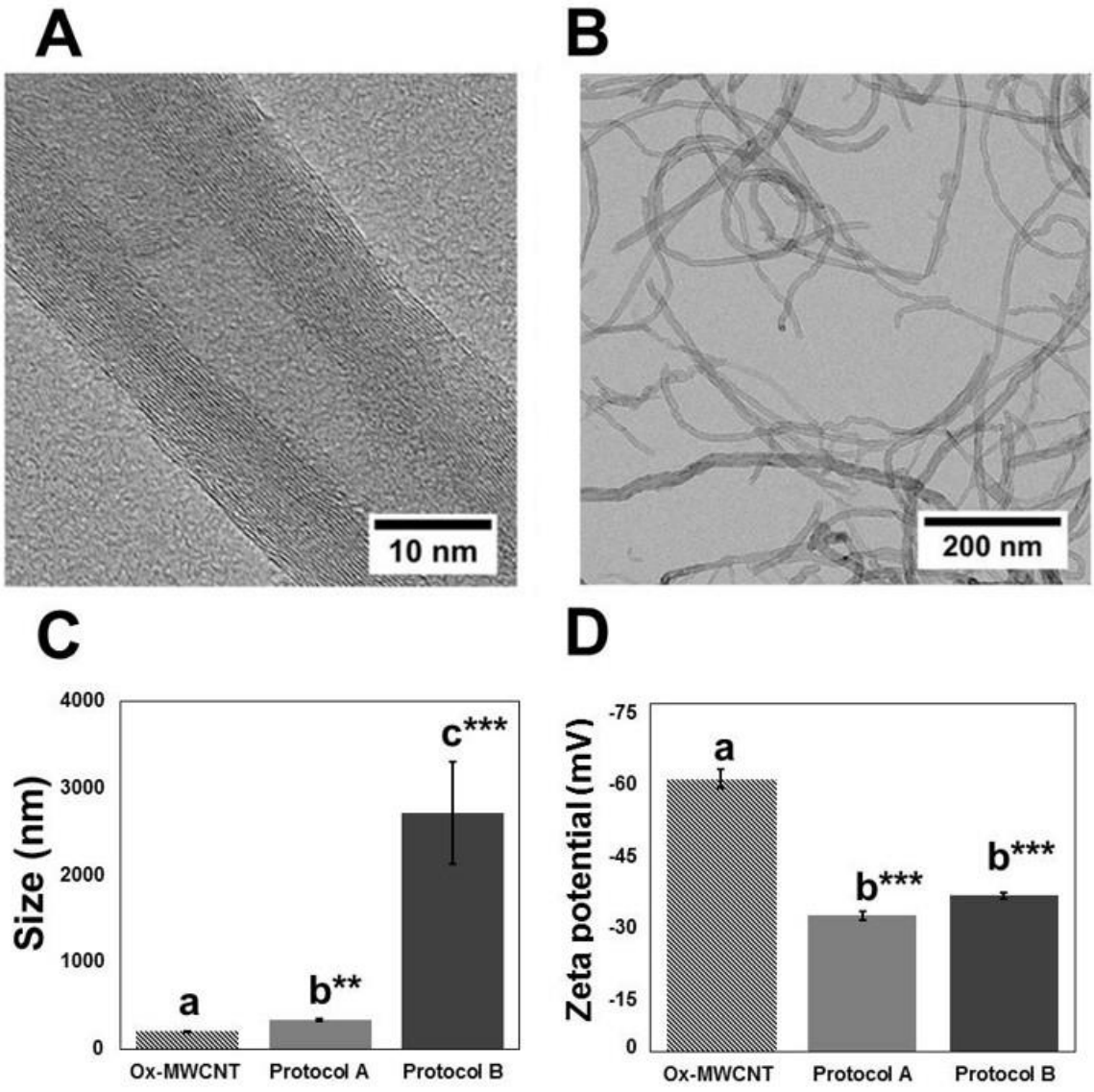

Figure 1. (**A and B)** TEM micrographs of oxidized multiwalled carbon nanotubes (ox-MWCNT); **(C)** Hydrodynamic particle size and **(D)** zeta potential of ox-MWCNT and complexes obtained by protocols A and B. Protocol A: incubation protocol A (medium+FBS and MWNTC for 30 minutes subsequent addition of $CdCl_2$ for more 30 minutes). Protocol B: incubation protocol B (MWNTC and $CdCl_2$ for 30 minutes and subsequent addition of medium+FBS for more 30 minutes).

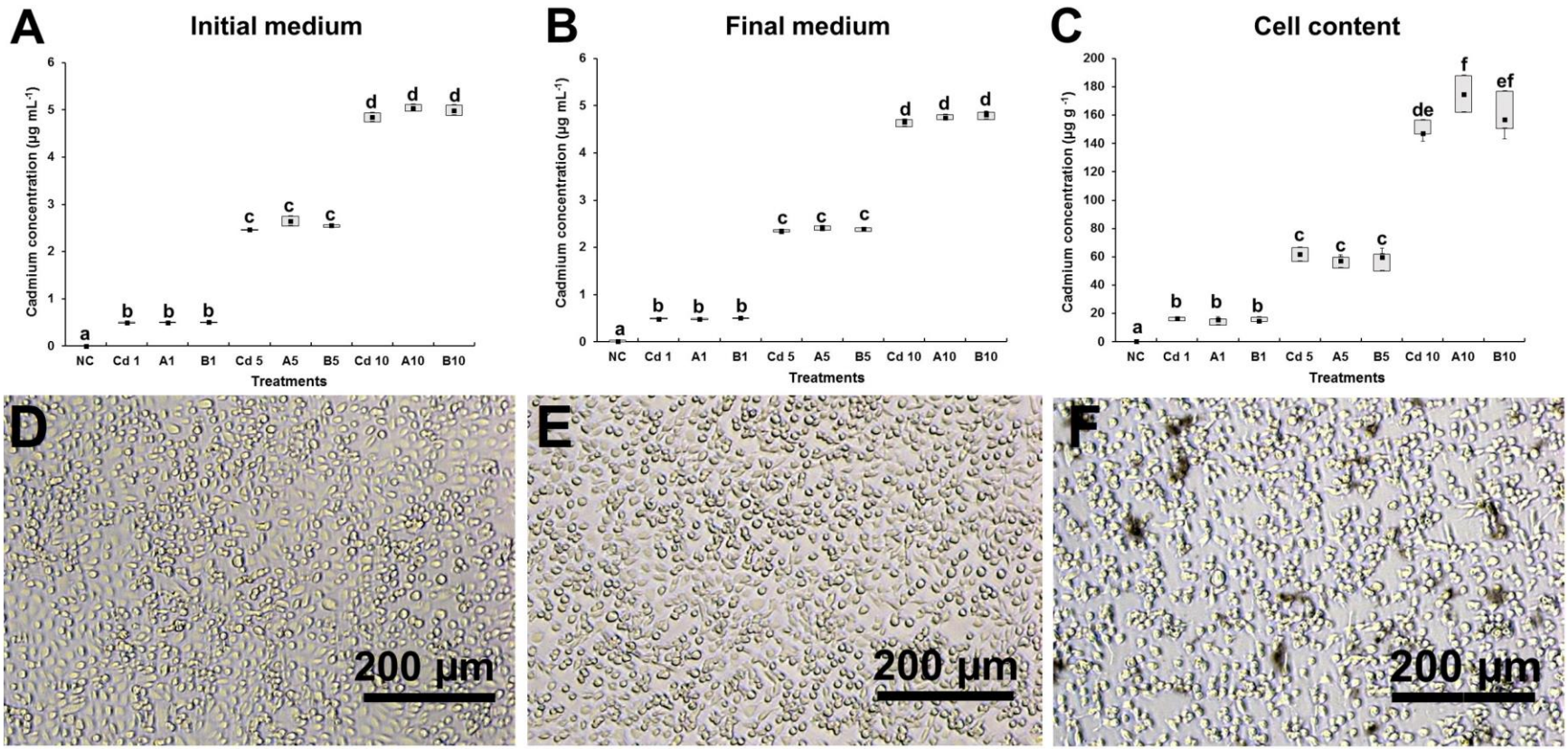

Figure 2. Cadmium (Cd) quantification. **(A)** Initial Cd content in culture medium (RPMI/L15) before cell exposure, **(B)** final concentration in the medium after cell exposure, **(C)** content in ZFL cells dry mass after treatments exposure for 24 hours. **(D, E and F)** ZFL morphology by light microscope of negative control and Protocol A and B at 10 µg mL^{-1} of $CdCl_2$ respectively. Different letters indicate differences between treatments (Kruskal-Wallis test, $p<0.05$). **NC:** negative control; **Cd1, Cd5, Cd10:** treatments with only $CdCl_2$ at 1, 5 and 10 µg mL^{-1}; **A1, A5, A10:** incubation protocol A at 1, 5 and 10 µg mL^{-1} (medium+FBS and ox-MWNTC for 30 minutes subsequent addition of $CdCl_2$ for more 30 minutes); **B1, B5, B10:** incubation protocol B at 1, 5 and 10 µg mL^{-1} (ox-MWNTC and $CdCl_2$ for 30 minutes and subsequent addition of medium+FBS for more 30 minutes).

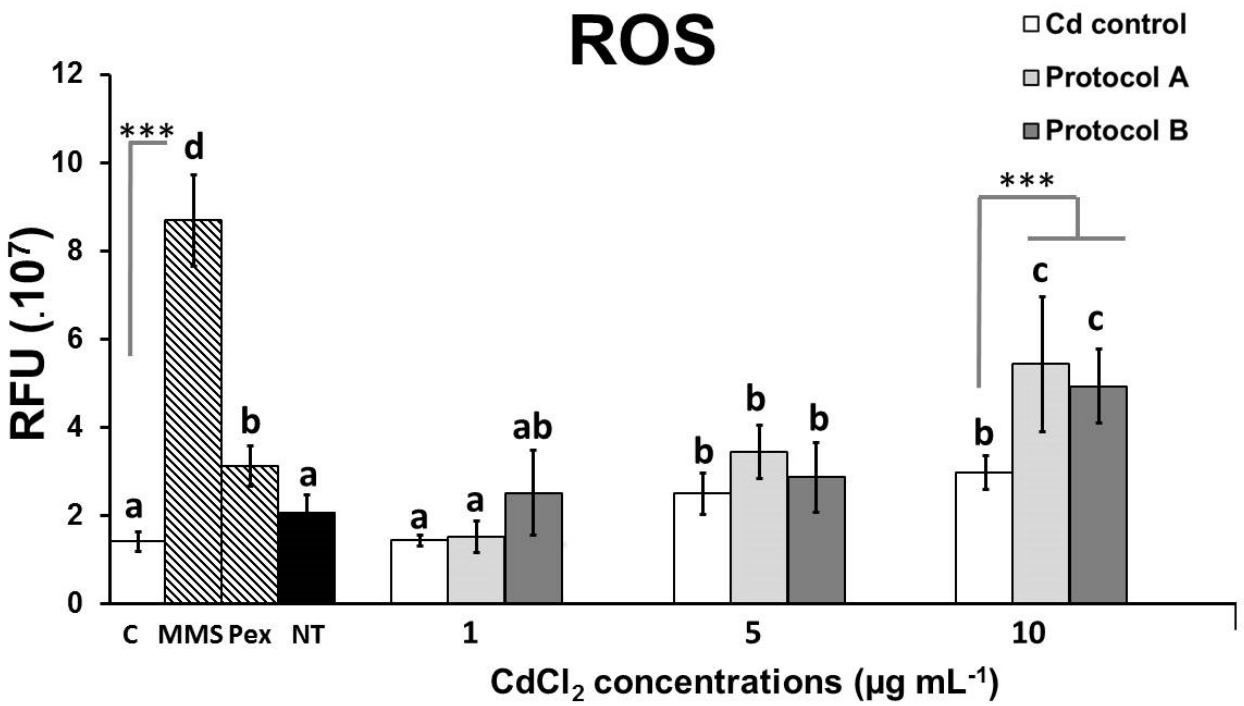

Figure 3. ROS production in ZFL cells after 24 hours exposure to the following treatments: negative control with PBS **(C)**; oxidized multiwalled carbon nanotube (5 µg mL^{-1}) (**NT)**; methyl methanesulfonate (**MMS,** 0.5 mM); hidrogen peroxide (**Pex,** 50 µM for 30min); treatments with only $CdCl_2$ (**Cd control**) and incubations with ox-MWNTC and $CdCl_2$ applying **Protocol A** and **Protocol B**. **RFU:** relative fluorescence unit. Different letters indicate differences between treatments by ANOVA/Tukey's test ($p < 0.05$). Asterisks indicates differences between the indicated treatments (***) $p < 0.01$, (**) $p < 0.05$.

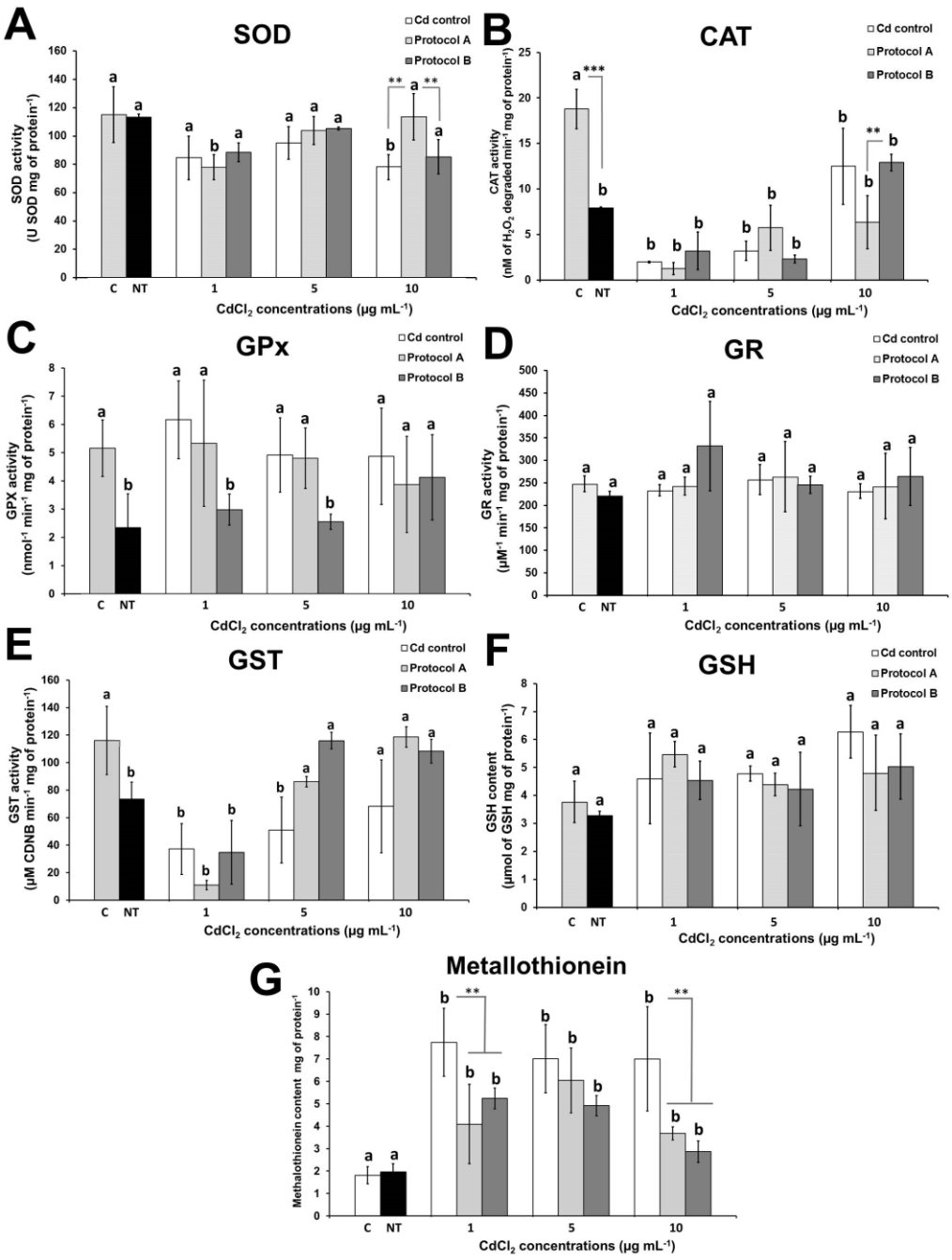

Figure 4. Antioxidant and biotransformation enzymes activity in ZFL cells exposed for 24 hours to $CdCl_2$ and ox-MWCNT and two co-exposure treatments (Protocol A nd B). **(A)** superoxide dismutase activity; **(B)** catalase activity; **(C)** glutathiona peroxidase activity; **(D)** glutathiona reductase activity; **(E)** glutathione S-transferase activity; **(F)** reduced glutathione content; **(G)** metallothionein content. **C:** negative control with PBS; **NT:** oxidized multiwalled carbon nanotube (5 µg mL^{-1}); **Cd control:** treatments with only $CdCl_2$; **Protocol A:** incubation protocol A (medium+FBS and ox-MWNTC incubated for 30 minutes and subsequent addition of $CdCl_2$ for more 30 minutes). **Protocol B:** incubation protocol B (ox-MWNTC and $CdCl_2$ for 30 minutes and subsequent addition of medium+FBS for more 30 minutes). Different letters indicate differences between treatments and negative control by ANOVA/Tukey's test ($p < 0.05$). Asterisks indicates differences between the indicated treatments (***) $p < 0.01$, (**) $p < 0.05$.

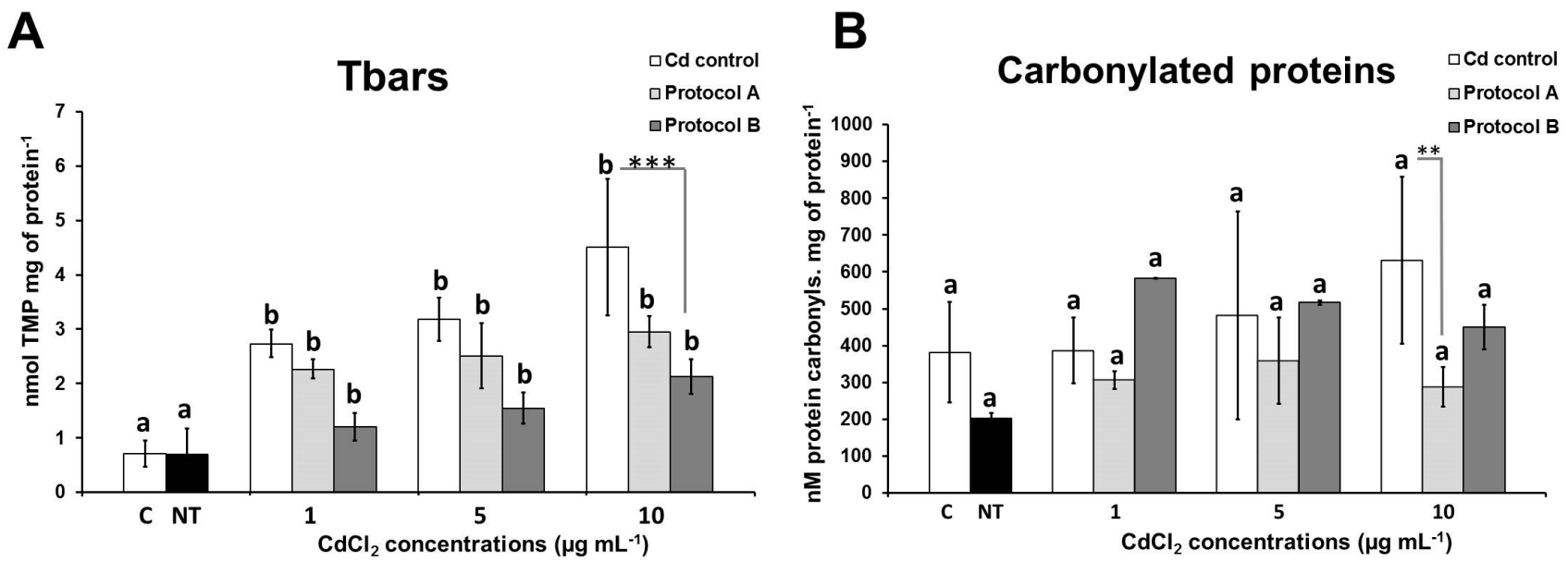

Figure 5. (A) Lipid oxidation by TBars method and **(B)** content of carbonilated proteins in ZFL cells exposed for 24 hours to two co-exposure treatments with $CdCl_2$ and ox-MWCNT in RPMI/L15 medium supplemented with serum. **C:** negative control with PBS; **NT:** ox-MWCNT, oxidized multiwalled carbon nanotube (5 µg mL^{-1}); **Cd control:** treatments with only $CdCl_2$; **Protocol A:** incubation protocol A (medium+FBS and ox-MWNTC incubated for 30 minutes and subsequent addition of $CdCl_2$ for more 30 minutes). **Protocol B:** incubation protocol B (ox-MWNTC and $CdCl_2$ for 30 minutes and subsequent addition of medium+FBS for more 30 minutes). Different letters indicate differences between treatments and negative control by ANOVA/Tukey's test ($p < 0.05$). Asterisks indicates differences between the indicated treatments (***) $p < 0.01$, (**) $p < 0.05$.

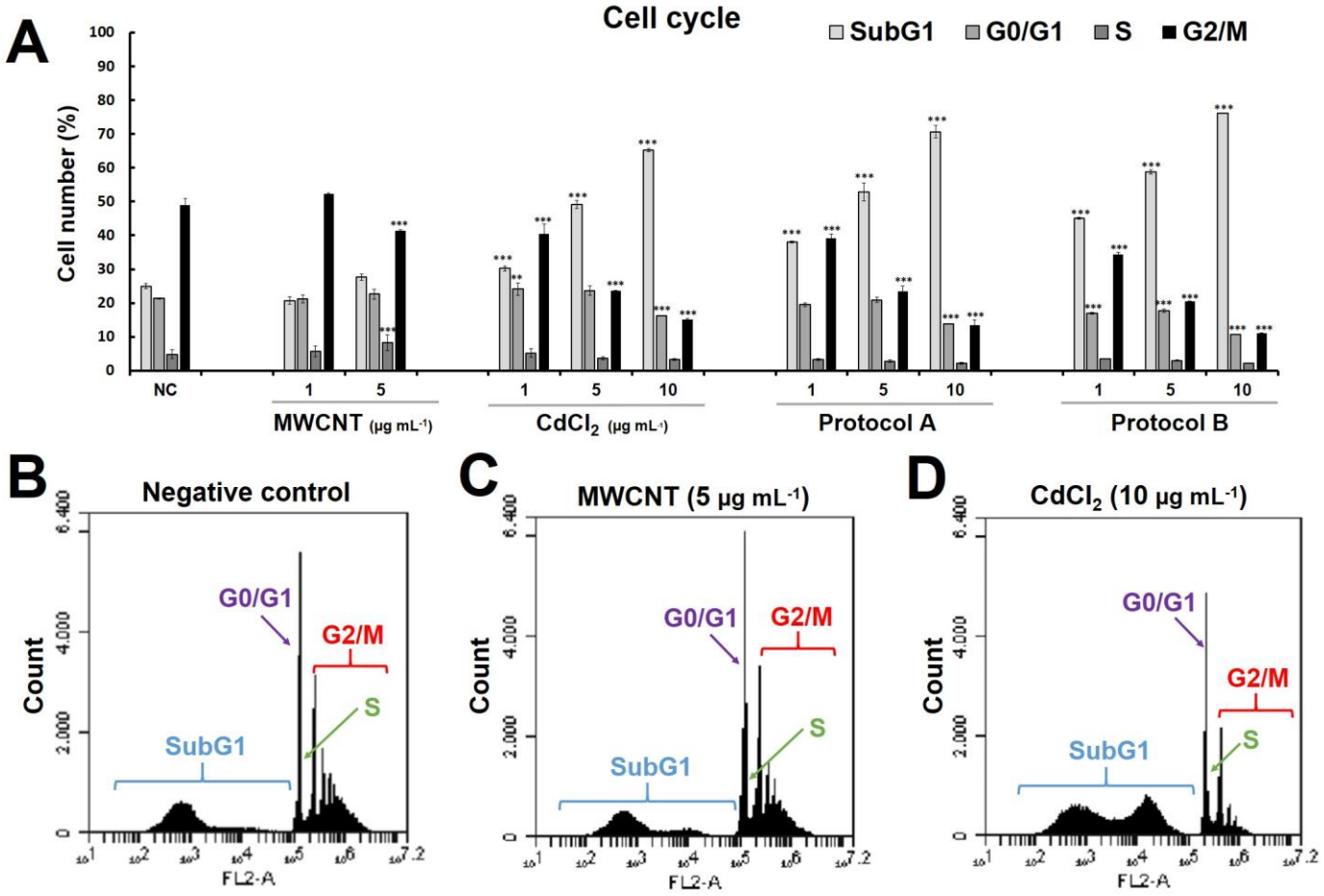

Figure 6. **(A)** Cell cycle results of ZFL cells exposed for 24 hours to two co-exposure protocols with $CdCl_2$ and ox-MWCNT in RPMI/L15 medium supplemented with fetal bovine serum; **(B,C and D)** cell population distribution graphic of negative control **(B)** ox-MWCNT, oxidized multiwalled carbon nanotube (5 µg mL^{-1}) **(C)**, and $CdCl_2$ (10 µg mL^{-1}) **(D)** evidencing cell populations in SubG1, G0/G1, S and G2/M. **NC:** negative control with PBS; **Protocol A:** incubation protocol A (medium+FBS and ox-MWNTC incubated for 30 minutes and subsequent addition of $CdCl_2$ for more 30 minutes). **Protocol B:** incubation protocol B (ox-MWNTC and $CdCl_2$ for 30 minutes and subsequent addition of medium+FBS for more 30 minutes). Asterisks indicates differences between negative control and indicated treatments by ANOVA/Tukey's test (***) $p < 0.01$, (**) $p < 0.05$.

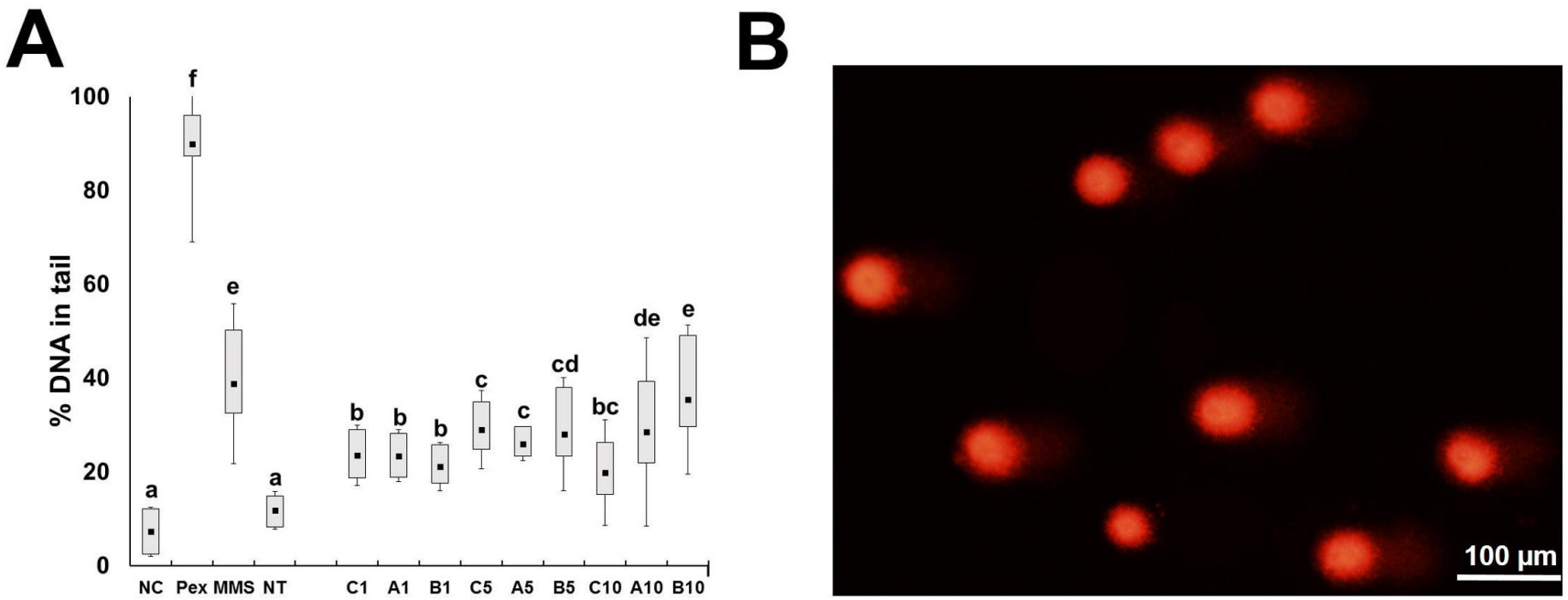

Figure 7. (A) Percentage of DNA in tail in comet assay results of ZFL cells exposed for 24 hours to two co-exposure treatments with $CdCl_2$ and ox-MWCNT in RPMI/L15 medium supplemented with serum. **(B)** Overall images of comets by fluorescence microscope with different DNA damage levels after software analysi **.** Different letters indicate differences between treatments (Kruskal-Wallis test, $p<0.05$). **NC:** negative control with PBS; **MMS**: methyl methanesulfonate (0.5 mM for 24h); **Pex:** hidrogen peroxide (50 µM for 30min); **NT:** ox-MWCNT, oxidized multiwalled carbon nanotube (5 µg mL^{-1}); **Cd control:** treatments with only $CdCl_2$; **Protocol A:** incubation protocol A (medium+FBS and ox-MWNTC incubated for 30 minutes and subsequent addition of $CdCl_2$ for more 30 minutes). **Protocol B:** incubation protocol B (ox-MWNTC and $CdCl_2$ for 30 minutes and subsequent addition of medium+FBS for more 30 minutes).

Tables

Table 1. Comet assay parameters (mean ± S.E.) in ZFL cells exposed for 24 hours to $CdCl_2$ and oxidized multiwalled carbon nanotubes (ox-MWCNT) and their interactions in RPMI/L15 medium supplemented with serum.

Treatments	**Comet area** (μM^2)	**OTM** (μM)
Negative control	17670 ± 744 ^{a}	4.7 ± 0.3 ^{a}
H_2O_2	44842 ± 4279 ^{f}	83.55 ± 0.7 ^{f}
Ox-MWCNT	20876 ± 699 ^{d}	7.9 ± 0.3 ^{b}
Cadmium control		
1 µg mL^{-1}	26199 ± 863 ^{e}	13.30 ± 0.7 ^{c}
5 µg mL^{-1}	25046 ± 826 ^{e}	18.10 ± 0.5 ^{e}
10 µg mL^{-1}	16064 ± 483 ^{a}	19.70 ± 0.6 ^{e}
Protocol A		
1 µg mL^{-1}	17376 ± 588 ^{a}	14.10 ± 0.4 ^{d}
5 µg mL^{-1}	17525 ± 531 ^{a}	16.30 ± 0.4 ^{e}
10 µg mL^{-1}	14350 ± 467 ^{b}	19.10 ± 0.9 ^{e}
Protocol B		
1 µg mL^{-1}	14866 ± 435 ^{b}	11.70 ± 0.3 ^{c}
5 µg mL^{-1}	15361 ± 502 ^{b}	15.10 ± 0.5 ^{d}
10 µg mL^{-1}	9537 ± 388 ^{c}	18.50 ± 0.9 ^{e}

Different letters represent differences between treatments in the same column (Kruskal Wallis, $p < 0.05$). **ox-MWCNT:** multiwalled carbon nanotube (5 µg mL^{-1}); **H_2O_2**: hidrogen peroxide (50 µM for 30min); **Cd control:** treatments with only $CdCl_2$; **Protocol A:** incubation protocol A (medium+FBS and MWNTC incubated for 30 minutes and subsequent addition of $CdCl_2$ for more 30 minutes). **Protocol B:** incubation protocol B (MWNTC and $CdCl_2$ incubated for 30 minutes and subsequent addition of medium+FBS for more 30 minutes); **OTM:** Olive tail moment.

5 CONCLUSÕES GERAIS

Este estudo fornece informações sobre a interação dos nanotubos de carbono oxidados de paredes múltiplas (ox-NTCPM) com o cádmio para o desenvolvimento de protocolos de coexposição e interação desses agentes. A partir das análises realizadas, esta tese possui as seguintes conclusões:

(I) Foi observada a formação de complexos diferentes de NTCPM-metal na presença ou ausência de soro bovino fetal (SBF) em meio de cultivo de células;

(II) A ordem das interações dos compostos em experimentos de coexposição *in vitro* leva à diferentes estabilidades dos NTCPM;

(III) A ordem das interações dos compostos nos experimentos testados não altera a composição de coroa de proteínas em ambos complexos NTCPM-metal estudados;

(IV) A ordem das interações dos compostos em experimentos de coexposição *in vitro* altera a taxa de adsorção de cádmio pelos NTCPM;

(V) O protocolo A, no qual os NTCPM interagem primeiro com SBF presente no meio de cultivo, e depois com metal é recomendado para ser adotado em estudos futuros, pois apresentou maior estabilidade em meio de cultivo;

(VI) O tratamento com apenas NTCPM não causou toxicidade nas células do fígado pelos testes convencionais do peixe-zebra, mas elevou as taxas de necrose e apoptose;

(VII) A interação entre os NTCPM e o cádmio em estudos *in vitro* afeta a internalização deste metal nas células e aumenta os efeitos genotóxicos;

(VIII) Os NTCPM promovem a depleção de catalase, glutationa peroxidase e glutationa S-transferase e alterações no ciclo celular com uma redução de células na fase G2/M;

(IX) Os NTCPM modulam os efeitos biológicos do Cd, por meio da sua influência na absorção de Cd pelas células e seus efeitos no bloqueio das enzimas

antioxidantes levando ao aumento dos danos genotóxicos promovidos pelo metal;

(X) Com base nos resultados, um efeito sinérgico foi observado dos agentes testados, também descrito na literatura de nanomateriais como “cavalo de tróia”.

O desenvolvimento de métodos e protocolos padronizados e reprodutíveis durante estudos de coexposição com poluentes ambientais são necessários para avaliar a ecotoxicidade dos nanomateriais. Além disso, as potenciais interações com os contaminantes existentes são de importância crucial para a avaliação dos impactos biológicos dos nanotubos de carbono em organismos aquáticos.

Este estudo tem implicações em futuras investigações sobre nanomateriais em ensaios de coexposição, e visa aprimorar os protocolos e os métodos empregados com intuito de contribuir com o entendimento sobre as interações de poluentes emergentes e contaminantes ambientais.

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