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FISIOLOGIA ANIMAL COMPARADA

EFEITO DOS NANOTUBOS DE CARBONO FUNCIONALIZADOS COM POLIETILENO GLICOL SOBRE A MEMÓRIA AVERSIVA E PARÂMETROS DE ESTRESSE OXIDATIVO NO HIPOCAMPO DE RATOS

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“A coisa mais bela que podemos
experimentar é o mistério. Essa é a fonte
de toda arte e ciência verdadeira."

Albert Einstein

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A todos, muito obrigada!

Sumário

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ACAP – capacidade antioxidante total contra radicais peroxil (do inglês *total antioxidant capacity against peroxyl radicals*)

ARE – elemento de resposta antioxidante (do inglês *antioxidant response element*)

BDNF – fator neurotrófico derivado do cérebro (do inglês *brain-derived neurotrophic factor*)

BHE – barreira hematoencefálica

CNT – nanotubos de carbono (do inglês *carbon nanotubes)*

GCL – glutamato cisteína ligase

GSH – glutationa reduzida

HO-1 – heme oxigenase-1

LTM – memória de longa duração (do inglês *long-term memory*)

LPO – peroxidação lipídica (do inglês *lipid peroxidation*)

MCC – medo condicionado contextual

MWCNT – nanotubos de carbono de paredes múltiplas (do inglês *multi-walled carbon nanotubes)*

NADPH – nicotinamida adenina dinucleotídeo fosfato (forma reduzida)

Nrf2 – fator nuclear eritróide 2 relacionado ao fator 2

PEG – polietilenoglicol

ROS – espécies reativas de oxigênio (do inglês *reactive oxygen species*)

RNS – espécies reativas de oxigênio (do inglês *reactive nytrogen species*)

SNC – sistema nervoso central

SWCNT – nanotubos de carbono de parede única (do inglês *single-walled carbon nanotubes)*

SWCNT-PEG – nanotubos de carbono de parede única funcionalizados com polietilenoglicol

TEM – microscopia eletrônica de transmissão (do inglês *transmission electron microscopy*)

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Resumo

Os nanotubos de carbono (CNT) são materiais promissores para aplicações biomédicas, especialmente no campo da neurociência. A capacidade destes nanomateriais de atravessar as membranas celulares e de interagir com as células nervosas traz a necessidade da avaliação dos potenciais efeitos deletérios dos CNT sobre o sistema nervoso. O objetivo deste trabalho foi investigar o efeito dos nanotubos de carbono de parede única funcionalizados com polietilenoglicol (SWCNT-PEG) sobre a memória aversiva de ratos e sobre parâmetros de estresse oxidativo no hipocampo. Para isso, ratos machos adultos foram submetidos à cirurgia estereotáxica para implante de cânulas no hipocampo e subseqüente infusão intrahipocampal das dispersões de SWCNT-PEG nas concentrações de 0,5; 1,0 e 2,1 mg/mL e da solução salina (grupo controle). A tarefa do medo condicionado contextual (CFC) foi utilizada para avaliar o efeito dos tratamentos nos seguintes estágios da memória de longa-duração: aquisição (infusão 30 min antes da sessão de treino), consolidação (infusão imediatamente após a sessão de treino), persistência (infusão 12 h após a sessão de treino) e evocação (infusão 30 min antes da sessão de teste). O teste da tarefa do CFC foi realizado 24 h após o treino nos grupos aquisição, consolidação e evocação e 7 dias depois no grupo persistência. Os tempos de exposição aos SWCNT-PEG foram de 30 min no grupo evocação, de 24 h nos grupos aquisição e consolidação e de 7 dias no grupo persistência. Os resultados deste trabalho evidenciaram um déficit na evocação da memória aversiva após a infusão das dispersões de SWCNT-PEG nas concentrações de 0,5 e 1,0 mg/mL. Este prejuízo da memória foi acompanhado pelo aumento da peroxidação lipídica (LPO) no hipocampo 30 min após a infusão das dispersões nas concentrações de 0,5 e 1,0 mg/mL. Decorridas 24 h, observou-se a diminuição da capacidade antioxidante total (ACAP) no hipocampo dos animais infundidos com as dispersões nas mesmas concentrações, embora não houve alterações na aquisição e consolidação da memória aversiva. A análise da distribuição de tamanho demonstrou que o tamanho médio das partículas de SWCNT-PEG foi maior na dispersão mais concentrada. Decorridos 7 dias da infusão das dispersões, observou-se o aumento da ACAP e do conteúdo da glutationa reduzida (GSH) no hipocampo em todas as concentrações do nanomaterial. A detecção dos SWCNT-PEG no hipocampo neste período indicou sua biopersistência no tecido. Em conjunto estes resultados demonstraram que o menor tamanho médio das partículas de SWCNT-PEG em dispersão foi determinante para sua resposta pró-oxidante inicial. A biopersistência do nanomaterial no hipocampo culminou no aumento das defesas antioxidantes em todas as concentrações estudadas. Foi sugerido que a maior dispersabilidade do nanomaterial no hipocampo ao longo do tempo pode ter permitido sua interação com as células e a indução das respostas antioxidantes pode constituir um importante mecanismo de adaptação à biopersistência dos SWCNT-PEG neste tecido.

Palavras-chave: Nanotubos de carbono; neurotoxicidade; estresse oxidativo; hipocampo; memória aversiva; biopersistência.

Abstract

Carbon nanotubes (CNT) are promising materials for biomedical applications, especially in the field of neuroscience. The ability of these nanomaterials to cross cell membranes and to interact with nerve cells brings the necessity to evaluate the potential deleterious effects of CNTs on the nervous system. The aim of this work was to investigate the effect of single-walled carbon nanotubes functionalized with polyethylene glycol (SWCNT-PEG) on the aversive memory of rats and on oxidative stress parameters in the hippocampus. For this purpose, adult male rats were subjected to stereotaxic surgery for implant of cannulae in the hippocampus with subsequent intrahippocampal infusion of SWCNT-PEG dispersions at concentrations of 0.5, 1.0 and 2.1 mg/mL and saline (control group). The contextual fear conditioning task (CFC) was employed to assess the effect of treatments on different stages of long-term memory: acquisition (infusion 30 min before training session), consolidation (infusion immediately after training session), persistence (infusion 12 h after training session) and retrieval (infusion 30 min before test session). The test of CFC task was performed 24 h after training in the acquisition, consolidation and retrieval groups, and 7 days after training in the persistence group. The exposure times to SWCNT-PEG were of 30 min for retrieval group, 24 h for acquisition and consolidation groups and 7 days for persistence group. The results of this work showed a deficit in retrieval of aversive memory after the infusion of SWCNT-PEG dispersions at concentrations of 0.5 and 1.0 mg/mL. This memory deficit was accompanied by the increase in lipid peroxidation (LPO) in hippocampus 30 min after the infusion of the dispersions at the same concentrations. After 24 h, there was a decrease of total antioxidant capacity (ACAP) in hippocampus of animals infused with the dispersions at 0.5 and 1.0 mg/mL, though there were no alterations on the acquisition and consolidation of aversive memory. The analysis of size distribution showed that the average particle size of SWCNT-PEG was higher in the dispersion with the highest concentration. After 7 days from the infusion of the dispersions, the increase in ACAP and in the content of reduced glutathione (GSH) was observed in the hippocampus at all the concentrations of the nanomaterial. The detection of SWCNT-PEG in hippocampus in this period indicated its biopersistence in the tissue. Altogether, these results demonstrated that the lowest average particle size of dispersed SWCNT-PEG was crucial for its initial pro-oxidant response. The biopersistence of the nanomaterial in hippocampus resulted in increased antioxidant defenses in all concentrations studied. It was suggested that the higher dispersibility of the nanomaterial in the hippocampus over time may have allowed its interaction with cells and the induction of antioxidant responses may be an important mechanism of adaptation to biopersistence of SWCNT-PEG in this tissue.

Keywords: carbon nanotubes; neurotoxicity; oxidative stress; hippocampus; aversive memory; biopersistence.

Introdução

A possibilidade de manipular a matéria na escala atômica a fim de se obter estruturas com características físicas particulares teve seu potencial revolucionário apresentado pelo físico Richard Feynman na reunião anual da *American Physical Society* em 1959 (Feynman, 1960). A pesquisa e o desenvolvimento da tecnologia em escala atômica, molecular ou macromolecular que culmina na criação e utilização de materiais ou dispositivos na escala nanométrica é denominada nanotecnologia (McNeil, 2005). O nanômetro (nm) corresponde à bilionésima parte do metro, ou 10^{-9} m, sendo designados "nanomateriais" os materiais de origem natural, incidental ou manufaturados que contêm, na distribuição de tamanho de partículas, 50% ou mais das partículas com uma de suas dimensões entre 1 e 100 nm (European Commission, 2011).

Nanomateriais de diferentes composições e arranjos atômicos vêm sendo estudados para o desenvolvimento de novas estratégias diagnósticas e terapêuticas, dentre os quais se destacam os nanomateriais de carbono, os *quantum dots*, as nanopartículas metálicas, os lipossomos, os dendrímeros e as nanopartículas poliméricas (Partha & Conyers, 2009; Raffa et al., 2010; Zhao et al., 2011; Chen et al., 2012). Estes nanomateriais podem interagir facilmente com as biomoléculas pois são menores do que a maioria das células e organelas e semelhantes em tamanho a grandes macromoléculas biológicas (McNeil, 2005), conforme ilustrado na figura 1. A capacidade dos nanomateriais de atravessar as barreiras celulares e interagir com receptores, ácidos nucleicos, fatores de transcrição e proteínas de sinalização pode ser utilizada para o desenvolvimento de ferramentas e dispositivos para monitoramento do funcionamento celular em condições fisiológicas e durante condições patológicas (Eckert et al., 2013).

A pesquisa e aplicação de nanomateriais e nanodispositivos para analisar e interagir com os sistemas biológicos a nível subcelular e molecular compreende um amplo campo científico denominado nanobiotecnologia (Torres et al., 2008). Os principais segmentos de pesquisa em nanobiotecnologia compreendem o desenvolvimento de ferramentas para monitoramento e detecção de eventos celulares e moleculares, a produção de biomateriais para engenharia e reparo de tecidos e agentes para diagnóstico por imagem e liberação de fármacos (Duncan & Gaspar, 2011). Uma área estritamente relacionada à nanobiotecnologia é a nanomedicina, que concentra as aplicações da nanotecnologia para o diagnóstico, prevenção e tratamento de doenças e tem impulsionado a pesquisa e produção de nanopartículas com perfil farmacocinético adequado às condições fisiopatológicas,

direcionamento para órgãos ou tecidos específicos e capacidade de translocação através das barreiras biológicas, especialmente a hematoencefálica (Shi et al., 2010; Bhaskar et al., 2010; Duncan & Gaspar, 2011).

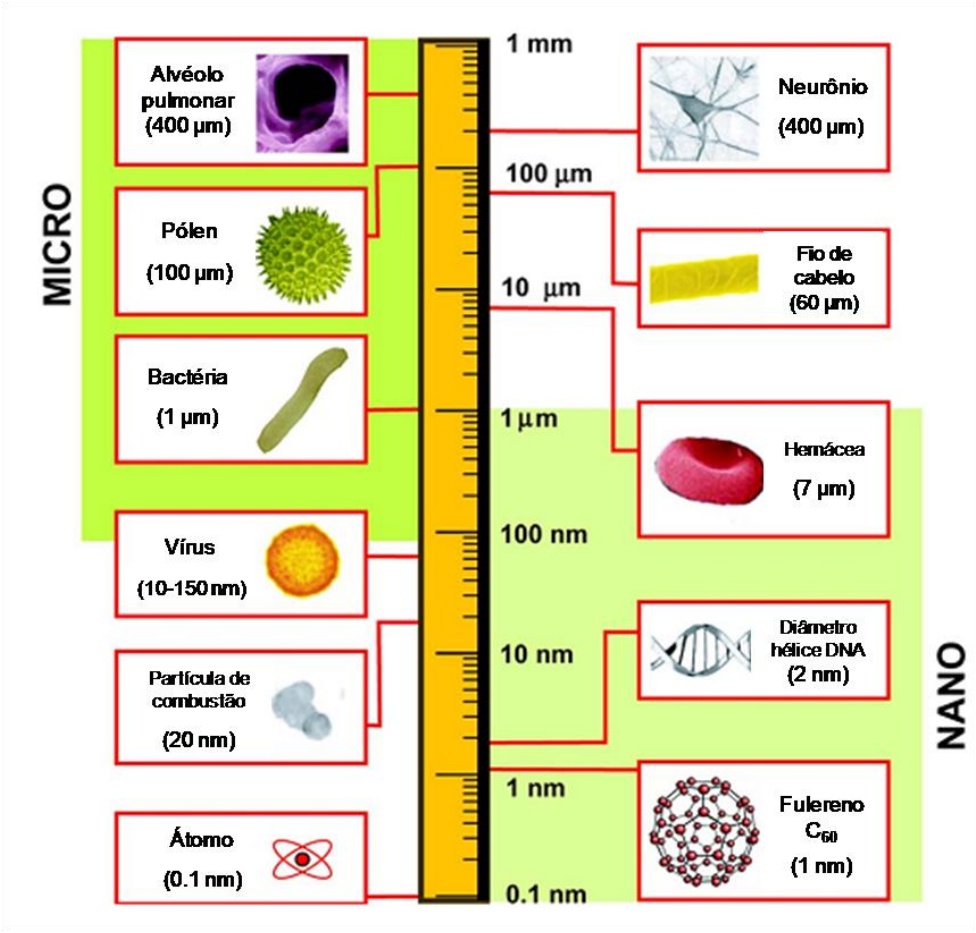

Figura 1. Representação comparativa de tamanho: da nano à microescala. Adaptado de Buzea et al., 2007.

Juntamente com as perspectivas de inovação na área biomédica, a nanotecnologia traz riscos potenciais à saúde humana. A toxicidade intrínseca das nanopartículas tem sido atribuída ao seu tamanho reduzido, elevada área de superfície por unidade de massa e alta reatividade da superfície (Auffan et al., 2009; Seaton et al., 2010). No entanto, é importante reconhecer que nem todas as nanopartículas são tóxicas, pois a toxicidade depende de diversos fatores, dentre os quais a composição química e a forma (Buzea et al., 2007; Drobne, 2007). Além dos fatores relativos aos nanomateriais, a ocorrência de efeitos tóxicos também depende das condições de exposição aos mesmos. As partículas ultrafinas do ambiente e as nanopartículas de engenharia podem atingir diferentes órgãos e tecidos do organismo humano através da inalação, ingestão ou contato com a pele, além da administração intencional para fins diagnósticos ou terapêuticos. A exposição a diferentes

nanopartículas pode causar potenciais efeitos adversos à saúde, os quais podem estar associados a processos patológicos crônicos e irreversíveis nos tecidos-alvo atingidos (Buzea et al., 2007), conforme representado na figura 2.

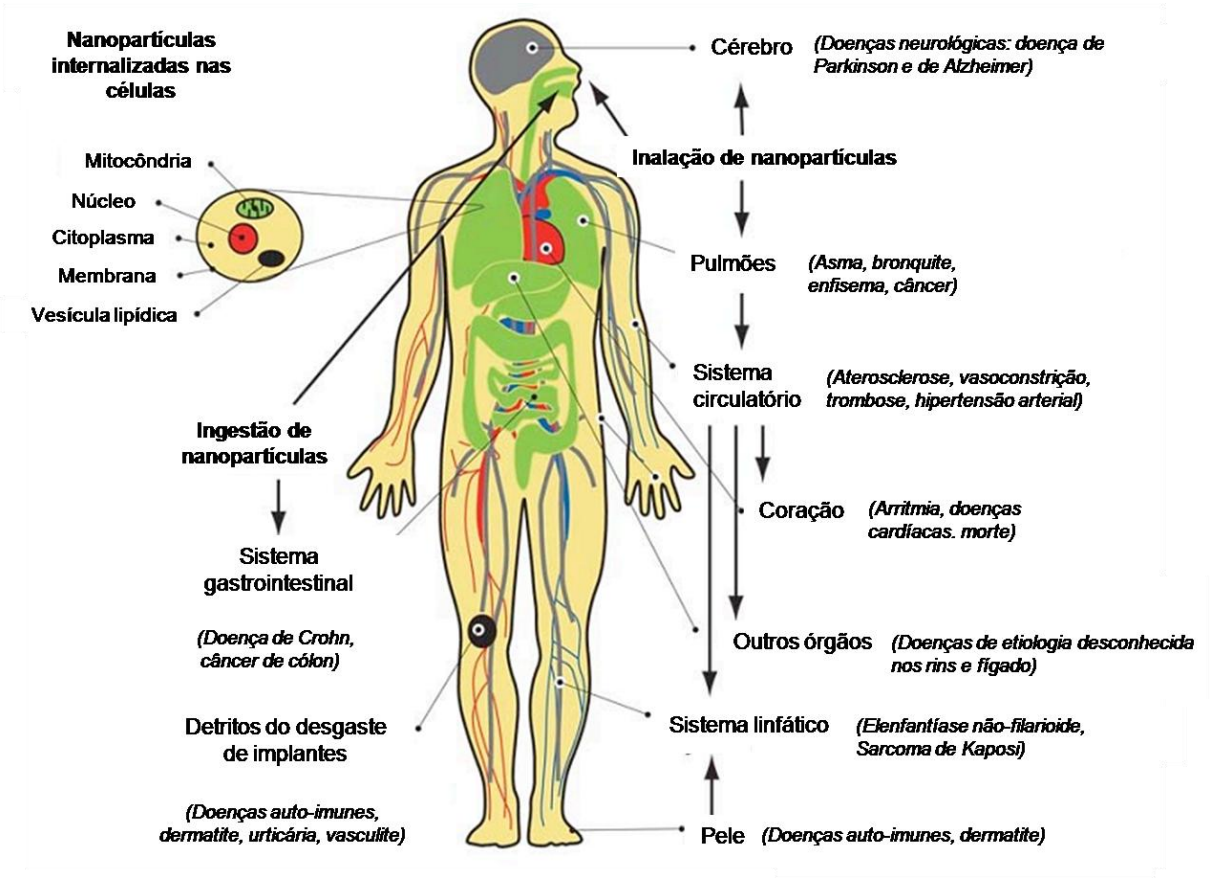

Figura 2. Principais vias de exposição às nanopartículas e possíveis doenças relacionadas aos órgãos afetados. Adaptado de Buzea et al., 2007.

A crescente produção e utilização de nanomateriais nas mais diversas áreas fez emergir nos últimos anos um novo ramo da toxicologia: a nanotoxicologia (Donaldson et al., 2004; Drobne et al., 2007; Oberdörster 2010). O maior desafio da nanotoxicologia consiste em identificar os principais fatores que podem ser utilizados para predizer a toxicidade de um novo nanomaterial. Com isso, novas estratégias e abordagens mais refinadas para caracterização e avaliação toxicológica dos nanomateriais devem ser desenvolvidas para atender às necessidades específicas da nanotecnologia (Donaldson et al., 2004; Drobne et al., 2007, Seaton et al., 2010).

Nanotubos de carbono

A descoberta dos fulerenos em 1985 por Kroto e colaboradores (Kroto et al., 1985) incentivou a busca por outras nanoestruturas de carbono e culminou, em 1991, com a síntese dos nanotubos de carbono (CNT, do inglês *carbon nanotubes*) (Iijima, 1991; Ball, 2001). Os fulerenos e CNT são nanoestruturas curvadas de carbono que tem o grafeno como elemento estrutural básico (Geim et al., 2007). Os CNT correspondem a folhas de grafeno enroladas em tubos de diâmetro nanométrico e comprimento na ordem de mícrons (Ajayan, 1999), os quais podem ser de parede única (SWCNT, do inglês *single-walled carbon nanotubes*), ou de paredes múltiplas (MWCNT, do inglês *multi-walled carbon nanotubes*), quando compostos por duas ou mais folhas de grafeno concêntricas (Iijima, 1991), conforme representado na figura 3. Os CNT vêm sendo amplamente estudados nos últimos anos para diversas aplicações, principalmente nas áreas da eletrônica, engenharia de materiais e biotecnologia (De Volder et al., 2013).

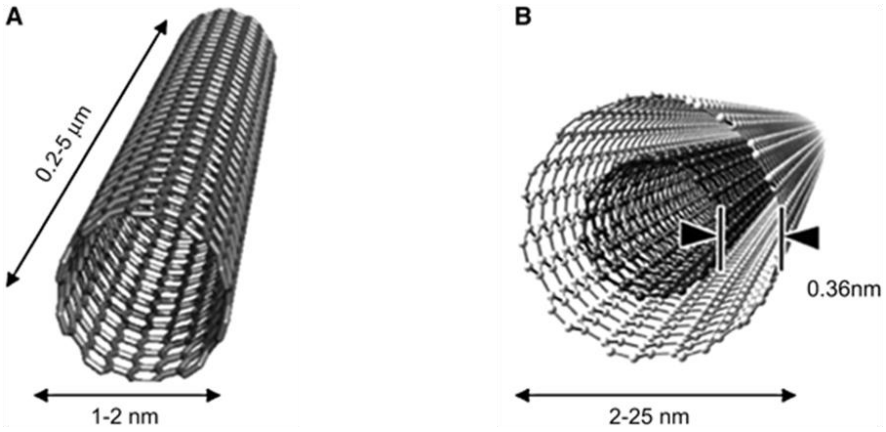

Figura 3. Representação dos nanotubos de carbono de parede única (A) e de paredes múltiplas (B) com suas dimensões aproximadas. Obtido de Reilly, 2007.

Os métodos comumente utilizados para a síntese dos CNT são a deposição química de vapor (CVD), a descarga de arco e a ablação a laser. A característica comum destes métodos é a adição de energia para uma fonte de carbono para produzir grupos de átomos de C individuais que se podem recombinar para gerando os CNT (Donaldson et al., 2006). A síntese por CVD utiliza gases de hidrocarbonetos como fontes de átomos de carbono e partículas de catalisadores metálicos como "sementes" para o crescimento dos CNT, que ocorre a temperaturas entre 500 e 1000 °C. A descarga de arco e a ablação a laser empregam precursores de carbono no estado sólido e envolvem vaporização a altas

temperaturas (milhares de graus Celsius). Estes métodos produzem CNT de alta qualidade, embora em pouca quantidade e com a formação de muitos subprodutos (Dai, 2002). Após a síntese, os CNT podem ser submetidos a diferentes processos de purificação para remoção das impurezas, geralmente compostas por partículas metálicas e carbono amorfo (Pumera 2007; Ge et al., 2008).

As propriedades mecânicas, elétricas e óticas especiais dos CNT têm despertado o interesse para suas aplicações na área biomédica, como novos substratos para crescimento e diferenciação celular (Jan & Kotov, 2007), sistemas de liberação de fármacos (Vashist et al., 2011) e de destruição seletiva de células (Kam et al., 2005), entre outras (Heister et al., 2013). A figura 4 apresenta diferentes formas de apresentação dos CNT e suas aplicações biomédicas. A baixa solubilidade dos CNT em muitos solventes representa uma limitação particularmente relevante para sua compatibilidade com os sistemas biológicos (Bianco et al., 2005), de modo que a funcionalização dos CNT é fundamental para sua utilização na área biomédica (Vardharajula et al., 2012). Diferentes grupos químicos podem ser incorporados à estrutura dos CNT a fim de atribuir novas características e funcionalidades a estes nanomateriais (Bianco et al., 2005).

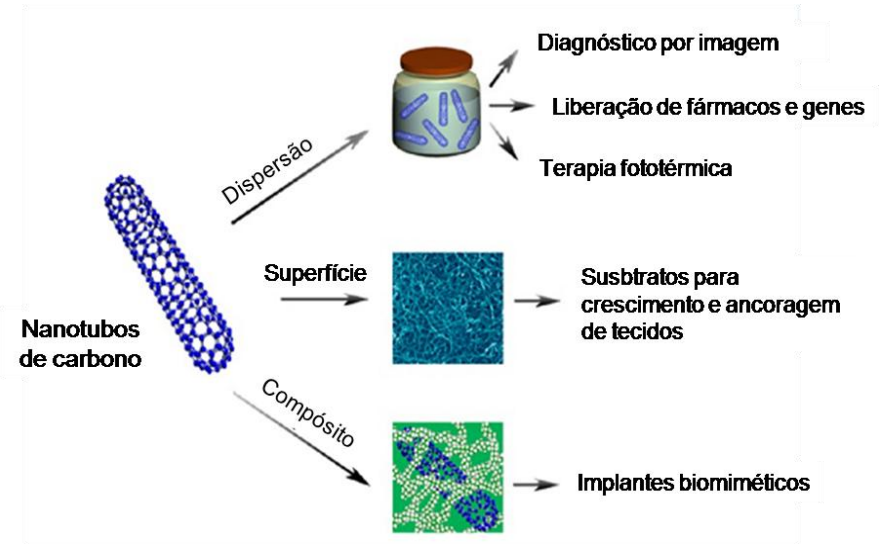

Figura 4. Potenciais aplicações biomédicas dos nanotubos de carbono. Adaptado de Heister et al., 2013.

A funcionalização dos CNT com polietilenoglicol (PEG), um polímero composto por unidades repetidas de etileno glicol, tem sido bastante utilizado devido sua flexibilidade e elevada hidrofilicidade. Além de aumentar a dispersabilidade em meios biológicos, a incorporação de moléculas de PEG na superfície dos CNT pode reduzir a

toxicidade e as respostas imunogênicas e prolongar a permanência destes nanomateriais na circulação sanguínea (Schipper et al., 2008; Liu et al., 2008; Bhirde et al., 2010; Bottini et al. 2011). A funcionalização dos CNT com PEG pode ainda ser utilizada para posterior conjugação com moléculas bioativas ou marcadores fluorescentes (Liu et al., 2009; Heister et al., 2013), conforme ilustrado na figura 5.

Estudos *in vitro* demonstraram que SWCNT funcionalizados com PEG podem ser incorporados pelas células por mecanismos distintos e se acumular em diferentes organelas, como nos lisossomos e mitocôndrias (Zhou et al., 2010), e no núcleo celular (Cheng, et al., 2008). Além das propriedades farmacocinéticas que impulsionam amplamente a utilização do PEG, alguns estudos têm demonstrado os efeitos neuroprotetores deste polímero após a injúria nervosa em mamíferos (Shi & Borgens, 2000; Luo & Shi, 2007; Koob et al., 2008). A administração local de SWCNT funcionalizados com PEG promoveu reparo axonal e recuperação funcional em ratos submetidos à lesão da medula espinal (Roman et al., 2011), indicando a ação neuroprotetora deste nanomaterial *in vivo*.

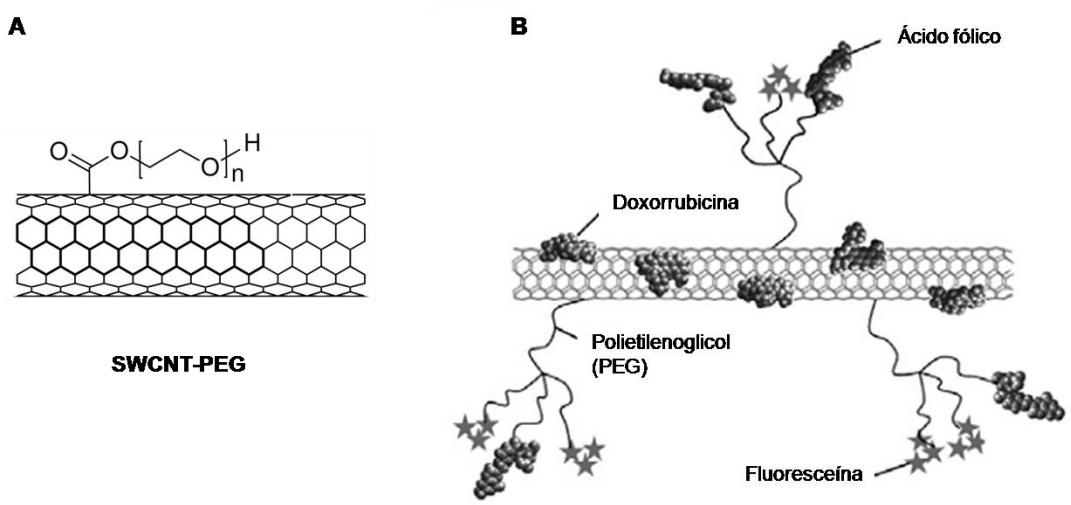

Figura 5. Funcionalização dos nanotubos de carbono. (A) Nanotubo de parede única funcionalizado com polietilenoglicol (SWCNT-PEG). (A) Obtido de http://www.sigmaaldrich.com. (B) Incorporação de moléculas biotivas e do agente fluorescente ao SWCNT-PEG. Adaptado de Heister et al., 2013.

A neurociência é um campo de estudo bastante promissor para as aplicações dos CNT (Malarkey & Parpura, 2010). Estudos *in vitro* demonstraram a biocompatibilidade de substratos contendo CNT com as células nervosas (Mattson et al., 2000; Liopo et al.,

2006; Galvan-Garcia et al., 2007), os quais podem modificar características morfo-funcionais de neurônios e astrócitos em cultura (Ni et al, 2005; Gottipati et al, 2012; Meng et al., 2013). As interações elétricas entre os CNT e as células nervosas vêm sendo investigada *in vitro* (Lovat et al, 2005.; Mazzatenta et al, 2007; Cellot et al. 2009), evidenciando a habilidade destes nanomateriais em aumentar a freqüência de eventos sinápticos e reforçar o número de sinapses das redes neurais (Cellot et al., 2011). Estes conjunto de evidências sustenta a possibilidade de se desenvolver nanodispositivos a base de CNT para registro de potenciais elétricos (Shoval et al., 2009), modulação da plasticidade sináptica (Cellot et al., 2011), restabelecimento de conexões entre neurônios (Fabbro et al., 2012) e regeneração neural (Voge & Stegemann, 2011).

Além das ações diretas dos CNT sobre as células nervosas e gliais, estes nanomateriais vêm sendo estudados como potenciais carreadores de fármacos para o sistema nervoso central (SNC). A capacidade de CNT quimicamente modificados de ultrapassar a barreira hematoencefálica (BHE) foi demonstrada para SWCNT ligados à acetilcolina, os quais se localizaram principalmente nos lisossomos das células neuronais de roedores e causaram disfunção mitocondrial e dano oxidativo nas altas doses (Yang et al., 2010). Um sistema para liberação de doxorrubicina e tratamento de glioma cerebral em ratos foi desenvolvido por Ren e colaboradores (2012) utilizando MWCNT modificados com proteínas capazes de reconhecer os receptores expressos na BHE e nas células tumorais. Estes estudos demonstram que a funcionalização dos CNT com diferentes grupos químicos ou moléculas biológicas foi capaz de adequá-los a objetivos específicos.

Uma vez que a superfície dos nanomateriais é determinante para as interações com os sistemas biológicos, o tipo e a densidade da funcionalização podem modificar as respostas tóxicas aos CNT (Sayes et al. 2006). Em uma revisão organizada por Madani e colaboradores (2013) estão listados os principais grupos funcionais utilizados na avaliação *in vivo* e *in vitro* da toxicidade destess nanomateriais, na qual é possível observar a existência de informações divergentes acerca da toxicidade dos CNT funcionalizados com PEG e ácido carboxílico (COOH). Além das diferenças relacionadas às abordagens experimentais empregadas, é preciso considerar que outros fatores, além das características da superfície, podem contribuir para a atividade biológica dos CNT, como a pureza, a estabilidade e o estado de aglomeração nos sistemas biológicos (Johnston et al., 2010), conforme apresentado na figura 6.

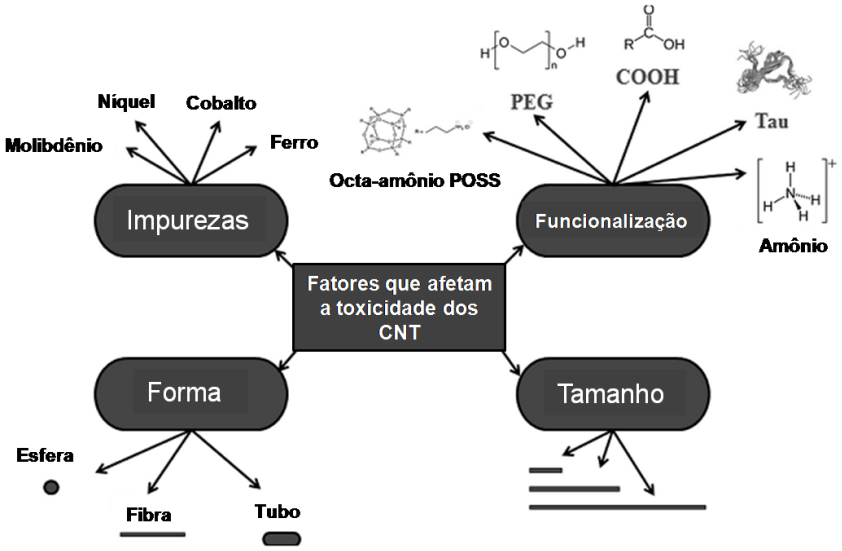

Figura 6. Fatores que afetam a toxicidade dos nanotubos de carbono (CNT). POSS, silsesquioxano oligomérico poliédrico; COOH, ácido carboxílico; PEG, polietilenoglicol. Adaptado de Madani et al., 2013.

Diversos estudos utilizando diferentes modelos biológicos, e abordagens experimentais, demonstraram o aumento na produção de espécies reativas de oxigênio (ROS, do inglês *reactive oxygen species*) após a exposição aos CNT, apontando a indução de estresse oxidativo como o principal mecanismo de toxicidade destes nanomateriais (Shvedova et al., 2008; Murray et al., 2009; Reddy et al, 2011; Zhang et al., 2011; Pichardo et al., 2012; Liu et al., 2014). Tal condição ocorre quando o aumento na quantidade dos agentes oxidantes, como as ROS e as espécies reativas de nitrogênio (RNS, do inglês *reactive nytrogen species*), excedem a capacidade dos sistemas antioxidantes perturbando a regulação da sinalização redox celular (Jones, 2006) ou causando dano oxidativo às biomoléculas (Valko et al., 2007). O dano oxidativo pode resultar em morte celular programada, ou apoptose, condição na qual há ativação de proteases específicas denominadas caspases e autodigestão controlada da célula (Orrenius et al., 2007).

A concentração intracelular das ROS e RNS é regulada pela atividade de sistemas de defesa antioxidante, os quais envolvem a ação de compostos com ação redox, que incluem a glutationa reduzida (GSH), a tiorredoxina e o ácido ascórbico (vitamina C), além de enzimas antioxidantes, como a superóxido-dismutase, a catalase e a glutationa peroxidase (Finkel & Holbrook, 2000). A GSH desempenha um papel central como antioxidante celular, pois além de atuar como um eficaz *scavenger* de ROS, contribui na manutenção do estado redox dos grupos tiol/dissulfeto das proteínas de outros antioxidantes nas suas formas reduzidas e funcionais (Jones, 2006). A enzima limitante

na síntese da GSH é a glutamato cisteína ligase (GCL) (Maher, 2006), cuja expressão gênica pode ser regulada por diferentes estímulos a fim de manter os níveis intracelulares de GSH. A queda no conteúdo da GSH em função de sua oxidação no ambiente celular pode indicar a ocorrência de estresse oxidativo (Schafer & Buettner, 2001).

Além de efeitos pró-oxidantes diretos, geralmente associados à presença de impurezas metálicas, foi demonstrado que os CNT são capazes de ativar as células inflamatórias, neutrófilos e macrófagos, que podem agir como potentes geradores de ROS através da ativação das enzimas mieloperoxidase e NADPH oxidase (Shvedova et al., 2012). A internalização celular dos CNT pelas células inflamatórias pode resultar na geração maciça de ROS com potenciais efeitos deletérios aos organismos expostos (Murray et al., 2009; Shvedova et al., 2008 e 2012) ou ainda na degradação oxidativa destes nanomateriais (Vlasova II et al., 2012). Alguns efeitos dos CNT relacionados ao estresse oxidativo estão ilustrados na figura 7.

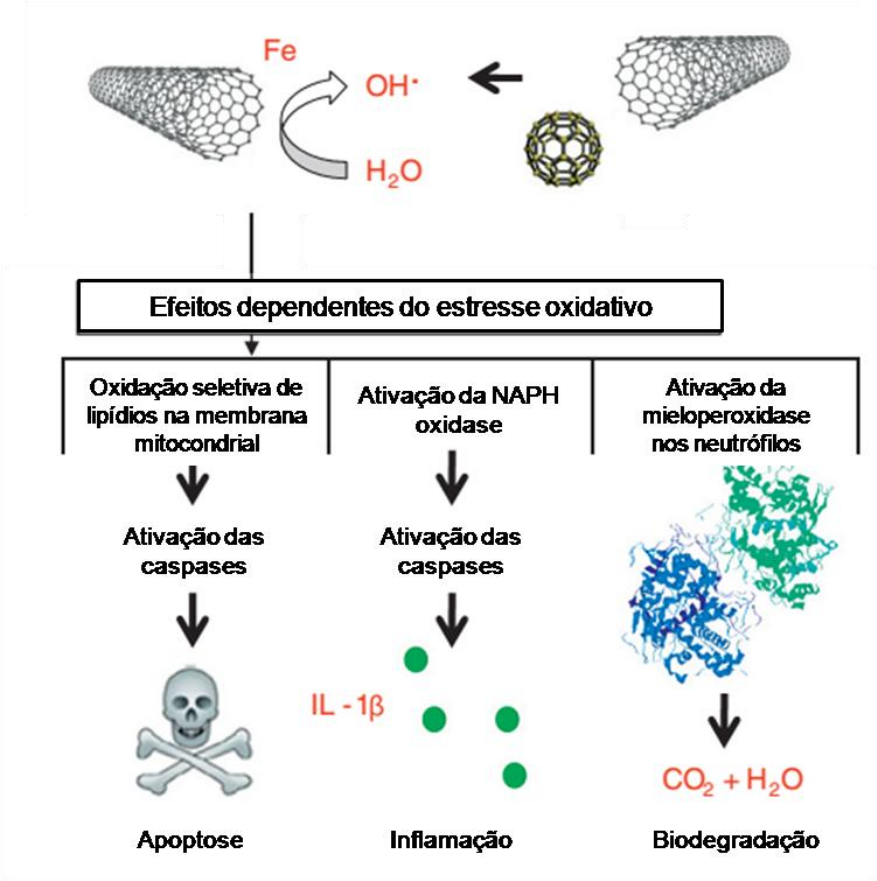

Figura 7. Potenciais mecanismos de toxicidade dos nanotubos de carbono (CNT) relacionados ao estresse oxidativo. Adaptado de Shvedova et al., 2012.

A indução de uma resposta inflamatória localizada acompanhada da degradação de MWCNT funcionalizados foi observada após a administração direta destes nanomateriais no córtex cerebral de ratos (Bardi et al., 2013). Embora seja proposto que a degradação dos CNT nos tecidos possa facilitar a eliminação e reduzir a toxicidade (Kagan et al., 2010), é possível que sejam gerados produtos de degradação, como hidrocarbonetos aromáticos oxidados (Allen et al., 2009), com potenciais ações tóxicas.

Neurotoxicologia comportamental

A maioria dos estudos acerca da neurotoxicidade do CNT foi conduzida em culturas neuro-gliais primárias e linhagens neuronais, com alterações relacionadas à inibição da proliferação e diferenciação celular (Belyanskaya et al, 2009; Meng et al 2013), inibição da secreção de catecolaminas (Gavello et al., 2013), indução de estresse oxidativo (Zhang et al, 2011; Wang et al, 2012) e alterações no funcionamento de canais iônicos (Park et al., 2003; Jakubek et al., 2009). Estes efeitos mediados pelos CNT podem comprometer a integridade e modificar o funcionamento do SNC, interferindo na regulação e manutenção de processos fisiológicos necessários a sobrevivência dos animais e sua adaptação ao ambiente, dentre os quais a memória. Neste contexto, a neurotoxicologia comportamental constitui um importante instrumento para a avaliação toxicológica *in vivo* de potenciais agentes neurotóxicos cujas ações a nível molecular, celular e genético podem culminar em alterações no comportamento dos animais expostos (Wallace, 2005; Levin et al., 2009).

A memória é a capacidade dos animais de adquirir, armazenar e evocar informações, permitindo as interações destes entre si e com o ambiente em que vivem (Izquierdo, 2011). O armazenamento das informações no SNC depende de alterações estruturais e funcionais nas conexões entre neurônios (sinapses), processo denominado plasticidade sináptica e que é dependente da experiência, ou seja, da atividade neuronal (Kandel, 2001; Izquierdo, 2011). A plasticidade sináptica envolve uma sincronia de eventos bioquímicos, como a ativação de receptores celulares e de vias de sinalização intracelular que culminam na síntese de novas proteínas, evento-chave para a consolidação das memórias de longa duração (LTM, do inglês *long-term memory*; Johansen et al., 2011), a qual persiste de horas a dias ou até mesmo anos (Izquierdo et al., 2006).

O processamento da memória envolve diferentes estágios (Abel & Lattal, 2001). Durante a aquisição, que corresponde ao aprendizado, ocorre a percepção dos estímulos

através das experiências sensoriais da interação com o ambiente. Na consolidação a informação adquirida sofre um processo gradual de filtragem e fixação e o traço da memória pode ser fortalecido ou enfraquecido, dependendo da relevância da informação (Izquierdo, 2011). Na evocação a informação consolidada é recuperada e expressada. Uma importante proteína envolvida no processamento da memória é o fator neurotrófico derivado do cérebro (BDNF, do inglês *brain-derived neurotrophic factor*) (Bekinstein et al., 2014). A indução do BDNF 12 horas após a aquisição da memória inicia uma cascata de eventos que culminam no remodelamento sináptico necessário para a persistência da memória, um evento-chave no armazenamento da LTM (Bekinschtein et al., 2008).

Uma estrutura do SNC que está diretamente relacionada à formação, consolidação e evocação das memórias episódicas é o hipocampo (Langston et al., 2010) uma complexa estrutura encefálica localizada bilateralmente no lobo temporal dos mamíferos (figura 8) e que desempenha um importante papel nas funções cognitivas destes animais, dentre as quais a memória episódica e a navegação espacial (Maren et al, 2013). Lesões no hipocampo de roedores produziram déficits na resposta de medo condicionado ao contexto, manifestada na forma de *freezing*, ou seja, a completa imobilidade do animal exceto pela manutenção dos movimentos respiratórios (Maren et al., 2013).

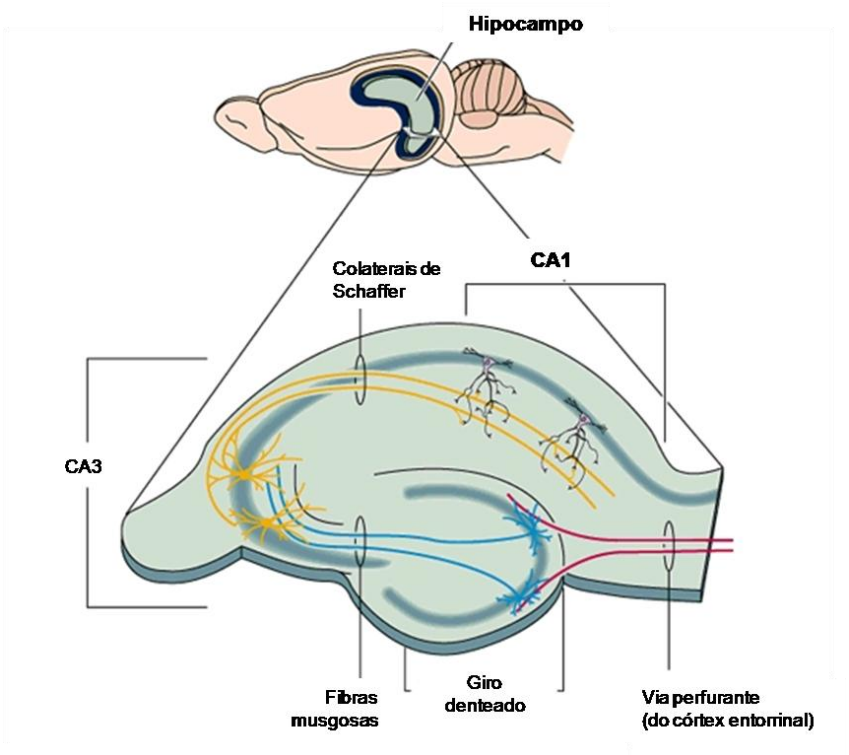

Figura 8. Anatomia do hipocampo de um roedor. CA, corno de Amon. Adaptado de http://www.arts.uwaterloo.ca/~bfleming/psych261/lec15no7.htm

Os testes comportamentais que envolvem o aprendizado associativo têm sido amplamente utilizados para o estudo do aprendizado e memória em animais de laboratório (Smith & Mizumori, 2006). O condicionamento contextual de medo, representado na figura 9, constitui uma ferramenta experimental muito utilizada em estudos farmacológicos, genéticos e bioquímicos da memória aversiva em roedores, em parte porque é uma forma altamente conservada do comportamento, exibido tanto nas condições de laboratório como nos ambientes naturais (Wehnerm & Radcliffe, 2004; Curzon & Rustay, 2009). O processamento da informação contextual durante o condicionamento ao contexto envolve a participação de distintas subregiões do hipocampo, especialmente da subregião denominada corno de Amon 1 ou CA1, cuja integridade é requerida na formação e evocação da memória de medo (Daumas et al, 2005; Hunsaker et al, 2009).

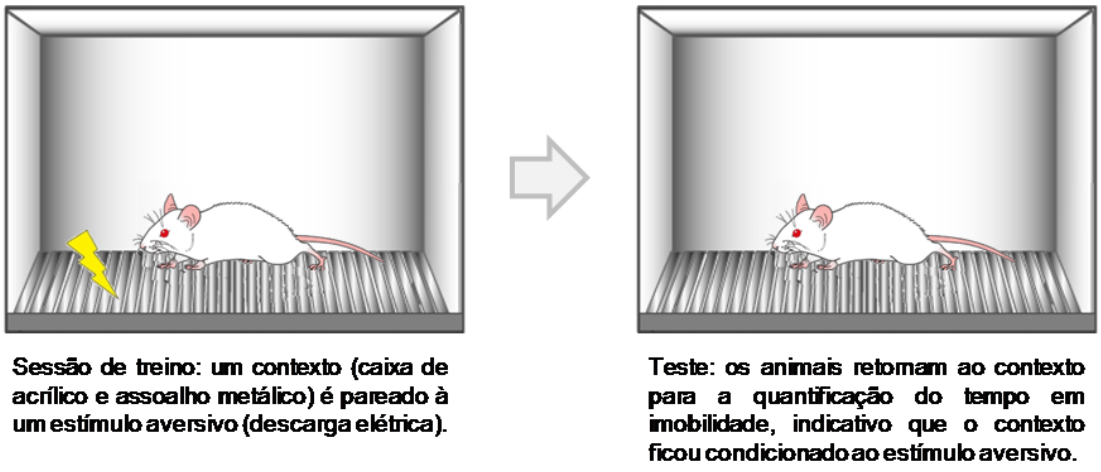

Figura 9. Tarefa do medo condicionado contextual. Adaptado de http://btc.bol.ucla.edu/fear.htm

A capacidade de muitos nanomateriais, inclusive os CNT, de causar estresse oxidativo (Buzea et al., 2007; Shvedova et al., 2012) é de especial importância na investigação das alterações neurológicas, tendo em vista que o estresse oxidativo está implicado na fisiopatologia de transtornos psiquiátricos e doenças neurodegenerativas (Agostinho et al, 2010; Maes et al, 2011; Kulak et al., 2013). O SNC é particularmente vulnerável ao estresse oxidativo devido seu elevado conteúdo de substratos oxidáveis, como ácidos graxos poliinsaturados e catecolaminas, e alta produção basal de ROS devido ao intenso metabolismo aeróbico (Halliwell, 1992; Sayre et al., 2008).

Considerando a habilidade de diversos nanomateriais em atingir o SNC (Hu & Gao, 2010), inclusive os CNT (Yang et al., 2010; Ren et al., 2012), é fundamental investigar as

possíveis modificações na estrutura ou na função do SNC decorrentes da exposição aos mesmos. Neste contexto, a avaliação comportamental pode ser um indicador relativamente sensível da toxicidade dos nanomateriais, visto que o comportamento envolve a integração de diferentes funções, dentre as quais a motora, sensorial e cognitiva, e também permite que sejam observadas alterações em outros sistemas que não o SNC (Tilson, 1987).

Objetivos

Objetivo geral

Avaliar os efeitos da administração intrahipocampal de nanotubos de carbono de parede única funcionalizados com polietilenoglicol (SWNT-PEG) sobre a memória aversiva de longa duração e parâmetros de estresse oxidativo no hipocampo de ratos.

Objetivos específicos

- Determinar os efeitos dos SWCNT-PEG sobre a aquisição, consolidação, evocação e persistência da memória aversiva de longa duração em ratos;

- Avaliar a produção de espécies reativas de oxigênio (ROS) e a ocorrência de lipoperoxidação (LPO) no hipocampo dos ratos infundidos com as dispersões de SWCNT-PEG nos diferentes tempos experimentais;

- Determinar a capacidade antioxidante total contra radicais peroxil (ACAP) do hipocampo dos animais após a infusão das dispersões de SWCNT-PEG nos diferentes tempos experimentais;

- Quantificar a atividade da enzima glutamato-cisteína ligase (GCL) e o conteúdo de glutationa reduzida (GSH) no hipocampo de ratos infundidos com as dispersões de SWCNT-PEG nos diferentes tempos experimentais;

- Determinar a biopersistência dos SWCNT-PEG no hipocampo após 1 e 7 dias da infusão das dispersões.

Resultados

Os resultados do estudo foram divididos em dois manuscritos, os quais compõem a seção de resultados desta tese.

Manuscrito 1: PEGylated carbon nanotubes affect memory retrieval and oxidative stress parameters in rat hippocampus

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PEGylated carbon nanotubes affect memory retrieval and oxidative stress parameters in rat hippocampus

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Keywords: PEGylated carbon nanotubes; neurotoxicity; hippocampus; oxidative stress; contextual fear memory; particle size.

Short Abstract: This work presented the effects of single-walled carbon nanotubes functionalized with polyethylene glycol (SWCNT-PEG) on the consolidation and retrieval of the contextual fear memory. The oxidative stress responses after 30 min and 24 h of the direct infusion of the SWCNT-PEG dispersions in the rat hippocampus were assessed. It was demonstrated that lower and intermediate but not higher concentrations of the nanomaterial were able to disrupt the memory retrieval and cause lipid peroxidation shortly after their infusion in the hippocampus.

Abstract

Carbon nanotubes (CNT) are promising materials for biomedical applications, especially in the field of neuroscience, so it is essential to evaluate the neurotoxicity of these nanomaterials. This work presented the effects of single-walled CNT functionalized with polyethylene glycol (SWCNT-PEG) on the consolidation and retrieval of long-term memory of rats and on oxidative stress parameters in the hippocampus. SWCNT-PEG were dispersed in water at concentrations of 0.5, 1.0 and 2.1 mg/mL and infused into the rat hippocampus immediately after the training and 30 min before the test of the contextual fear conditioning task, resulting in the exposure times of 24 h and 30 min, respectively. The results showed that the short exposure to SWCNT-PEG impaired the aversive memory retrieval and caused lipid peroxidation in the hippocampus. Such response was transient and overcome by the antioxidant defenses mobilization after 24 h, when there was a decrease in the total antioxidant capacity. These effects occurred at low and intermediate but not at higher concentration of the SWCNT-PEG, suggesting that the biological response may be related to the particle size of the nanomaterial since it was found a concentration-dependent increase in the particle size of SWCNT-PEG in the dispersions.

Introduction

The potential of carbon nanotubes (CNT) to reestablish synaptic connections and promote nervous system repair has stimulated many studies in the recent years (Roman et al., 2012; Fabbro et al., 2012; Parpura et al., 2013). Notwithstanding, little is known about the effects of CNT in the brain of living organisms. Studies with animals exposed systemically to SWCNT revealed distinct effects of this nanomaterial on the central nervous system, from the absence of effects upon behavior and the induction of the tissue protection against oxidative damage (da Rocha et al., 2013; Boyle et al., 2014) to cognitive deficits and increased oxidative stress levels (Liu et al, 2014).

It should be considered that systemic administration of CNT may result in different biodistribution and toxicity profiles, according to the type of nanomaterial, the route of administration and animal model used (Liu et al., 2008; Lacerda et al., 2008; Yang et al., 2012). Thus, an alternative to study the interactions between CNT and neural tissue is the direct administration of the nanomaterial into the brain, as allowed by stereotaxic injection. Using this methodology, Bardi et al (2013) demonstrated that ammonium functionalized multi-walled CNT (MWCNT) were internalized into neurons and microglia, causing a transient neuroinflammatory response in the murine cortex.

The present study aimed to evaluate the effects of SWCNT functionalized with polyethylene glycol (SWCNT-PEG) on the hippocampus, one of the most sensitive brain regions to oxidative stress (Wang & Michaelis, 2012), which is required for the processing of the contextual memory (Daumas et al., 2005; Wiltgen et al., 2010). Here, were demonstrated the effects of SWCNT-PEG dispersions infused in the dorsal hippocampus on the consolidation and retrieval of the contextual fear memory and on the oxidative stress parameters. The physicochemical characteristics of SWCNT-PEG dispersions were described to determine their contribution to the observed effects.

Material and Methods

Chemicals

SWCNT-PEG, Tris-HCl (Trizma® hydrochloride), ethylenediaminetetraacetic acid (EDTA), 2,2'-azobis(2-methylpropionamidine) dihydrochloride (ABAP), glutathione (GSH), adenosine triphosphate (ATP), glutamate, borate, serine, cysteine, cumene hydroperoxide (CHP) and xylenol orange were purchased from Sigma-Aldrich (St.

Louis, MO, USA) and KCl, $MgCl_2$ and $FeSO_4$ from Labsynth (Diadema, SP, Brazil). 2',7'-dichlorofluorescein-diacetate (H_2DCF-DA) and naphthalene-2,3-dicarboxyaldehyde (NDA) were purchased from Molecular Probes (Eugene, OR, USA) and Biuret protein assay kit from Doles (Goiânia, GO, Brazil).

Preparation of SWCNT-PEG dispersions

SWCNT-PEG (PEG Mw = 600 Da, Sigma-Aldrich-652474-Lot MKBC 9435) were synthesized via the electric arc discharge method. For the production of stable dispersions in water, several steps of mechanical disintegration and centrifugation were employed, using a procedure adapted from Kalinina et al. (2011). Initially, SWCNT-PEG was dispersed in water at a concentration of 3 mg/mL by ultrasonication for 48 h using a bath sonicator (Cole Parmer, model 08895-50, ~ 40 kHz). The mixture was subsequently placed in a high-shear rotor-stator mixer (IKA Labortechnik, Ultra-Turrax T8) for 1 h 30 min and centrifuged (Eppendorf, 5417C) for 30 min at 5,000 g to remove large particles and agglomerates. The supernatant was then carefully removed, diluted four times and placed in an ultracentrifuge (Sorvall, Ultra Pro 80) for 1 h at 170,000 g to remove the excess of unbound PEG, non-tubular carbon and other impurities. In the next step, the resulting pellet was resuspended in water to give a dispersion concentration of around 2 mg/mL and subjected again to bath sonication for 4 h, shear mixing for 30 min, and centrifugation for 30 min at 5,000 g. Dilutions of the final dispersion were made by adding ultrapure water to reach the concentrations of 0.5 and 1.0 mg/mL and subjecting the dispersions to bath sonication for 30 min.

Physico-chemical characterization

As-received SWCNT-PEG and the dispersion were characterized by low resolution (LR) and high resolution (HR) transmission electron microscopy (TEM). LRTEM were performed on FEI Tecnai G2-Spirit 120 kV and HRTEM on FEI Tecnai G2-20 SuperTwin 200 kV microscopes. The metallic impurities were determined by energy dispersive X-ray spectroscopy (EDS). Raman spectra were obtained using a modular Raman system (Horiba JobinYvon, RMS-550) with an excitation wavelength of 514 nm. The final concentration of SWCNT in the dispersion was spectrophotometrically evaluated by performing optical absorption measurements using a Shimadzu UV-vis-NIR spectrophotometer UV-3600 over the wavelength range of 190 to 1100 nm and taking as reference a sample of known concentration. Zeta potential of the SWCNT-PEG dispersions at 0.5, 1.0 and 2.1 mg/mL was determined by electrophoretic light

scattering (Zetasizer Nano ZS® system, Malvern Instruments), diluting the original dispersion 10 times after 30 min of bath sonication, filtering through 0.45 µm membrane and adjusting the ionic strength to 10 mM using NaCl. Size distribution, polydispersity index (PDI) and hydrodynamic particle diameter (z-average) were analyzed by dynamic light scattering (DLS, Zetasizer Nano ZS® system, Malvern Instruments). Z-average and PDI were calculated by means of cumulant analysis. The hydrodynamic diameter of SWCNT-PEG measured by DLS corresponds to the equivalent hydrodynamic diameter of a sphere that has the same translational diffusion coefficient as the SWCNT-PEG nanoparticles. Thus, the values obtained do not correspond to a single dimension (length or diameter) of the nanomaterial, but rather to a combined value of the both dimensions.

Animals

Wistar male rats (2-3 months of age; weight 250-320 g) were obtained from a breeding colony of the Universidade Federal do Rio Grande (RS, Brazil) and randomly selected and housed in polycarbonate cages (5 rats per cage) with free access to water and food. The animals were kept under a 12 h light/dark cycle and constant temperature (23 ± 1 ˚C) and frequently manipulated to avoid neophobia. All animal procedures were approved by the Ethics Committee for Animal Use of the Universidade Federal do Rio Grande (FURG; Permit Number: P029/2011).

Intrahippocampal infusions

After a week of acclimation the animals were submitted to stereotaxic surgery for the bilateral implant of cannula on the dorsal hippocampus. The surgical procedure was performed as described by de Aguiar et al (2013) and Parfitt et al (2012). After 48-72 h of recovery from surgery, rats were distributed randomly in groups according to the treatments (n=10-12). SWCNT-PEG dispersions at 0.5, 1.0 or 2.1 mg/ml or 0.9 % NaCl solution (control group) were infused using 27-gauge injection needles inserted into each guide cannula and connected by polyethylene tubing to a Hamilton microsyringe. Infusions were all at a volume of 1 µl and were carried out first on one side and then on the other. The infusions were performed in two different times: immediately after the training session or 30 min before the test of contextual fear conditioning (CFC) task, resulting in the exposure times of 24 h and 30 min, respectively.

Contextual fear conditioning task

Contextual fear conditioning (CFC) apparatus consist in a box (dimensions 28 x 26 x 23 cm) of three aluminium walls, Plexiglas front wall and floor made of a series of a parallel stainless steel bars connected to shock scrambler deliver apparatus (shock generator, Insight Scientific Equipments, Brazil). The CFC task was performed as previously described by Bekinschtein et al (2007). Briefly, on the training session were applied three consecutive single electric foot-shocks (1 sec duration, 0.7 mA intensity) at 10 sec intervals. Test session was performed twenty-four hours after training, when the animals were checked for freezing (absence of any movement except that required for breathing) during 5 minutes. Both training and test sessions were performed between 8:00 and 12:00 a.m. and after each session the floor and walls of CFC apparatus were cleaned with 70 % ethanol. Results were expressed as the percentage of time spent in freezing in a 5 min period).

Tissue dissection and samples preparation

At the end of the CFC test, all animals were killed by decapitation and their hippocampi were quickly dissected and stored at -80 ºC until use except for ROS measure, for which they were immediately homogenized (1:5 w/v) in 40 mM ice-cold Tris-HCl buffer (pH 7.4) For antioxidant capacity against peroxyl radicals (ACAP), glutamate cysteine ligase (GCL) activity and glutathione (GSH) content analysis, hippocampi were thawed on ice and homogeneized in buffer containing 100 mM Tris-HCl, 2 mM EDTA and 5 mM $MgCl_2$ (pH 7.75). Hippocampal homogenates were centrifuged at 20,000 g, 4 ˚C, for 20 min. The supernatants had their total proteins content measured by the Biuret method using microplate absorbance reader (BioTek LX 800) and the final protein concentration was adjusted to 3 mg/mL. For LPO analysis, hippocampi were homogenized (1:15 w/v) in 100% ice-cold methanol and centrifuged at 1000 g for 10 min at 4˚ C. Supernatants were used in all assays.

Reactive oxygen species generation

Reactive oxygen species (ROS) were measure using the compound H_2DCF-DA as previously employed in brain tissue (Aguiar et al., 2008; Galhardi et al., 2009). Briefly, the samples were placed in reaction buffer (pH 7.2) containing 200 mM KCl, 30 mM HEPES, 1 mM $MgCl_2$ and 16 μM H_2DCFDA. Formation of the oxidized fluorescent product dichlorofluorescein (DCF) was monitored with a fluorescence microplate reader (485 nm excitation/520 nm emission; Victor 2, Perkin Elmer) with readings every 5 min for 30 min. ROS generation was calculated by integrating the fluorescent units (FU)

along the time of measurement and after fitting the data to a second order polynomial function. ROS generation was expressed in FU.

Total antioxidant capacity against peroxyl radicals

Determination of total antioxidant capacity against peroxyl radicals (ACAP) was performed following the methodology described by Amado et al (2009). The assay employed ROS quantification with H_2DCF-DA (40 µM final concentration) in hippocampus samples treated or not with a peroxyl radical generator (ABAP, 4 mM). DCF formation was recorded by fluorescence microplate reader (485 nm excitation/520 nm emission; Victor 2, Perkin Elmer) with readings every 5 min for 30 min. The inverse of relative difference between ROS area with and without ABAP was considered as a measure of antioxidant capacity.

Glutathione reduced content and glutamate-cysteine ligase activity

Glutathione reduced (GSH) content and glutamate-cysteine ligase (GCL) activity were determined according White et al (2003). The method was based in the reaction of naphthalene-2,3-dicarboxyaldehyde (NDA) with GSH or γ-glutamylcysteine (γ-GC) to form cyclic products that are highly fluorescent. Further details about the assay can be found in da Rocha et al (2009). NDA-GSH fluorescence was measured (472 nm excitation/528 nm emission) in a fluorescence microplate reader (Víctor 2, Perkin Elmer). GSH content was expressed in nM of GSH and GCL activity in nM of GCL h^{-1}.

Lipid peroxidation measurement

Lipid peroxides levels were measured by the spectrophotometric ferrous oxidation/xylenol orange (FOX) modified method as previously described by Monserrat et al (2003), with adjustments in time of incubation and sample dilution according de Aguiar et al (2008). The method is based on the oxidation of Fe(II) under acidic conditions and quantification of lipid hydroperoxides using CHP 0.1 mM as a standard. The assay was performed on a microplate using 5 µL of sample volume or 5 µL of methanol as negative control. Absorbance (550 nm) was determined using a microplate reader (BioTek LX 800). Lipid peroxidation (LPO) was expressed by CHP nM per gram of wet tissue.

Statistical analysis

Statistical analysis and graphs creation were carried out in GraphPad Prism 5.0 (GraphPad Software, Inc). Hydrodinamic size, polydispersity index and zeta-potential data were analyzed by one-way statistical analysis of variance (one-way ANOVA) followed by Tukey pos-hoc test. Biochemical and behavioral data were analyzed by one-way ANOVA followed by Newman-Keuls multiple comparisons test. Normality and variance homogeneity were verified as ANOVA assumptions. $P<0.05$ was considered as statistically significant. The results of biochemical assays were normalized to percentage of the control group.

Results

SWCNT-PEG characterization

LRTEM revealed that the SWCNT are completely enveloped by the polymer, forming large aggregates that were effective separated in fibers after ultrasound bath combined with high shear mixer treatment (Fig. 1A and 1B). Besides these fibers, were observed impurities and isolated polymer plates which were not completely removed by the centrifugation (Fig. 1C). EDS analysis revealed that metallic impurities were mainly composed of nickel and yttrium. By HRTEM we observed that SWCNT are functionalized in the form of bundles, containing, on average, from 5-10 tubes, making it impossible for the sample to be completely broken in the form of individual tubes (Fig. 1C). These beams have very heterogeneous lengths mostly between 600 nm and 800 nm. The Raman spectra of SWCNT-PEG dispersion after the dispersion protocol (Fig. 1D) confirmed that not all tubes were functionalized, i.e. the functionalization occurs mainly on the SWCNT bundle surfaces rather than on individual nanotubes since a well-defined and intense G band and radial breathing mode (RBM) were observed. By the intensity of light absorption at 700 nm the final concentration of the sample was estimated in 2.1 ± 0.2 mg/mL. Size distribution analysis revealed a bimodal particle size distribution (Fig. 2A), with the lower-sized mode between 10 and 1000 nm and the larger particle sizes mode between 1000 and 10000 nm. The heterogeneous particle size distribution observed in this study was confirmed by the PDI analysis (Fig. 2B), which provides a measure of the broadness of the particle size distribution. Here we found PDI values around 0.4, with the lower PDI value for SWCNT-PEG dispersion at 0.5 mg/mL. The z-average revealed a concentration dependent increase in the particle size of SWCNT-PEG dispersed in water (Fig. 2C). The zeta potential values were between -

23 and -33 mV (Fig. 2 D) and did not correlate with the mean particle size of SWCNT-PEG dispersions.

Behavioral parameter

SWCNT-PEG dispersions infused immediately after the training session did not affect the aversive memory consolidation, as shown in Fig. 3A. In this experimental time, the animals treated with SWCNT-PEG dispersions presented a similar time spent in freezing to the control group, revealing that these animals could discriminate the fear context and expressed it through freezing behavior. On the other hand, the infusions of SWCNT-PEG dispersions at 0.5 and 1.0 mg/mL made 30 min before the test session cause a deficit in the retrieval of fear memory (Fig.3B), such impairment was not observed in the group that received SWCNT-PEG dispersion at 2.1 mg/mL.

ROS generation

There was a significant increase in ROS generation in the hippocampus 24 h after the infusion of SWCNT-PEG dispersions at 0.5 mg/mL, as shown in Fig. 4A. This increase was around 23% above the average of control group. There was no statistical difference in ROS generation between SWCNT-PEG dispersions and control group after 30 min of the treatments infusion (Fig. 4B).

ACAP determination

Hippocampal ACAP was significantly reduced 24 h after infusion of SWCNT-PEG dispersions at 0.5 and 1.0 mg/mL (Fig. 5A). The decrement in ACAP was 35% and 30% in the groups receiving the dispersions of 0.5 to 1.0 mg/mL, respectively, when compared to control group. There was no difference in ACAP among the experimental groups 30 min after the treatments (Fig. 5B).

GSH content and GCL activity

The results of GSH content and GCL activity are presented in Figure 6. GSH content of the hippocampus was unaltered in both 30 min and 24 h post injection SWCNT-PEG groups (Fig. 6A and 6B). Regarding GCL activity, a significant increase was found 24 h after the infusion of SWCNT-PEG dispersions at 0.5 and 1.0 mg/mL (Fig. 6C). There was no difference in GCL activity among the treatments 30 min after the infusion (Fig. 6D).

LPO measure

Lipid hydroperoxides levels in the hippocampus were unaltered in SWCNT-PEG treated groups 24 h after the infusion (Fig. 7A). However, 30 min after the infusion of SWCNT-PEG dispersions at 0.5 and 1.0 mg/mL there was a significant increase in LPO (Fig. 7B). Comparing to control group, the increase was around 34% and 39% for SWCNT-PEG dispersions at 0.5 mg/mL and 1.0 mg/mL, respectively.

Discussion

Neurobehavioral studies are of great importance in toxicological research since they can assess the functional integrity of the nervous system and detect a latent damage and the mechanisms of action of chemically induced neurotoxicity (Tilson, 1987; Dorman, 2000). There are few studies showing the neurobehavioral changes from exposure to CNT, despite the growing interest in developing nanodrugs and nanodevices for applications in neuroscience. Here we used stereotactic surgery to infuse SWCNT-PEG dispersions at different times and to evaluate their effects in two important stages of the fear memory processing.

The results of this work showed a significant increase in lipid peroxidation in the hippocampus 30 min after infusion of the SWCNT-PEG dispersions at 0.5 and 1.0 mg/mL, while there was a deficit in memory retrieval. This oxidative damage could account, at least partly, for the impairment of the fear memory retrieval. However, with 24 h of exposure to SWCNT-PEG, stand out the mobilization of antioxidant defenses, as demonstrated by the decrease in the ACAP and the raise in GCL activity, and there was no increment in GSH content of the hippocampus. These modifications were followed by the increase in ROS generation only in the lowest concentration of the SWCNT-PEG dispersion, which did not result in oxidative damage or impairment in memory consolidation.

Oxidative stress is postulated as the underlying mechanism of CNT toxicity (Shvedova et al., 2012). The occurrence of oxidative stress after CNT exposure has been associated to the presence of metallic catalyst residues (Pulskamp et al., 2007; Shvedova et al., 2012; Aldieri et al., 2013; Meng et al., 2013) that can be partially removed by different purification protocols (Pumera et al., 2007; Ge et al., 2008). However, we observed that metal nanoparticles remained in the SWCNT-PEG even after the dispersion protocol and were mainly composed of nickel and yttrium, two catalysts commonly used in the synthesis of CNT by electric arc (Laplaze et al., 2002).

These remaining impurities persist in the SWCNT-PEG dispersions in a non-bioavailable form, as metal nanoparticles surrounded by multiple graphitic layers and linked covalently to the beams. Thus, we concluded that the biological response to SWCNT-PEG may not to be attributed to these metallic impurities, since modifications in memory retrieval and oxidative stress parameters did not occur in the dispersion of SWCNT-PEG at the higher concentration.

The biological response observed in this study occurred at lower and intermediate but not in the higher concentration of the SWCNT-PEG dispersions, which presented a concentration-dependent increase in their hydrodynamic diameter. Thus, it is reasonably to propose that the effects of the SWCNT-PEG in the hippocampus can be related to others physicochemical characteristics of the nanomaterial, as size, agglomeration state and surface chemistry (Jin et al., 2009; Kolosnjaj-Tabi et al., 2010; Sohaebuddin et al., 2010). In order to know the characteristics of CNT in the dispersions, it has been employed the DLS technique, which provides information on the hydrodynamic properties of CNT, as particle diameter and size distribution (Chappell et al., 2009, Adeleye & Keller, 2014).

The particle size distribution of SWCNT-PEG dispersions presented a bimodal curve that can indicate the presence of both well dispersed SWCNT-PEG and the its agglomerates, as previously proposed for different CNT dispersions (Nadler et al., 2008; Krause et al., 2010; Frømyr et al., 2012). It was observed that PDI values and z-average increased with the concentration of SWCNT-PEG in the dispersions, suggesting a higher agglomeration tendency with the increased concentration of CNT in the dispersions. The concentration dependent increment in the particles size was previously demonstrated for nanomaterials of different compositions (Sohaebuddin et al., 2010).

The association between the particle/agglomerate size and the toxicity of CNT is controversial, probably due to differences in nanomaterial composition and size or cell types used in the studies (Sohaebuddin et al., 2010). It has been demonstrated that micron-size agglomerates of SWCNT are more cytotoxic (Wick et al., 2007; Belyanskaya et al., 2009; Kolosnjaj-Tabi et al., 2010) than better dispersed SWCNT bundles. On the other hand, micron-sized agglomerates of CNT at high concentrations can reduce their toxicity by decreasing the reactive surface area and possibly limiting their translocation and interaction with toxicity targets in cells (Coccini et al., 2010). In agreement with these observations, Hirano et al (2008) reported a concave dose response curve in cell viability of MWCNT-exposed cells and showed that the

nanomaterial was agglomerated at higher concentration, which may have reduced the MWCNT-cell interaction and prevented the toxicity of this nanomaterial.

The effects of SWCNT-PEG on the memory retrieval and oxidative stress parameters observed in this study occurred in the dispersions that presented the mean particles sizes of 88.1 and 104.8 nm, but not in the dispersion with the mean particle size of 108.9 nm. Although the difference of mean particle size between the dispersions at 1.0 and 2.1 mg/mL seems to be too small to justify the distinct biological response, it should be noted that the dispersion at higher concentration showed the most heterogeneous particle size distribution which could result in less small-sized particles that effectively interacted with hippocampal cells, therefore reducing the early neurotoxic effect of SWCNT-PEG dispersion at 2.1 mg/mL. So, the adverse interactions of the SWCNT-PEG with cellular targets may be facilitated by smaller particles present in greater proportion in the dispersions in the dispersions at lower and intermediate concentrations.

Besides the particle size, the surface functionalization of CNT may be an important factor in modulating their toxicity, since it can modify their water solubility, agglomeration state and surface charge (Vardharajula et al, 2012). The zeta potential is an indicator of the magnitude of the electrostatic interactions between colloidal particles and it measurement has been used to discuss the density of acidic sites on the surface of CNT (Sun et al., 2002; Kim et al., 2003). The negative zeta potential values of SWCNT-PEG dispersions indicate the presence of many carboxyl acid groups (-COOH) unreacted.

The functionalization of CNT with carboxyl groups was associated to decreased toxicity and bioactivity of these nanomaterials (Hamilton et al., 2013), however, it was also demonstrated that PEGylation of the free carboxyl groups mitigated the cytotoxicity of shorter MWCNT with high surface carboxyl density (Singh et al., 2012). In this study, the dispersions of SWCNT-PEG at 1.0 and 2.1mg/mL presented similar zeta potential values, but had distinct effects on memory retrieval and oxidative stress. Thus, it was not clear if the more negative zeta potential is affecting the dispersibility of SWCNT-PEG and such contributions to the biological response.

In conclusion, we found that SWCNT-PEG caused distinct behavioral and biochemical changes in the hippocampus according to the time of the infusion and the particle size of the SWCNT-PEG agglomerates. These results may have important implications for the safety assessment of CNT, particularly with regard to the influence

of the physicochemical characteristics and temporal variations in biological response to the nanomaterial exposure.

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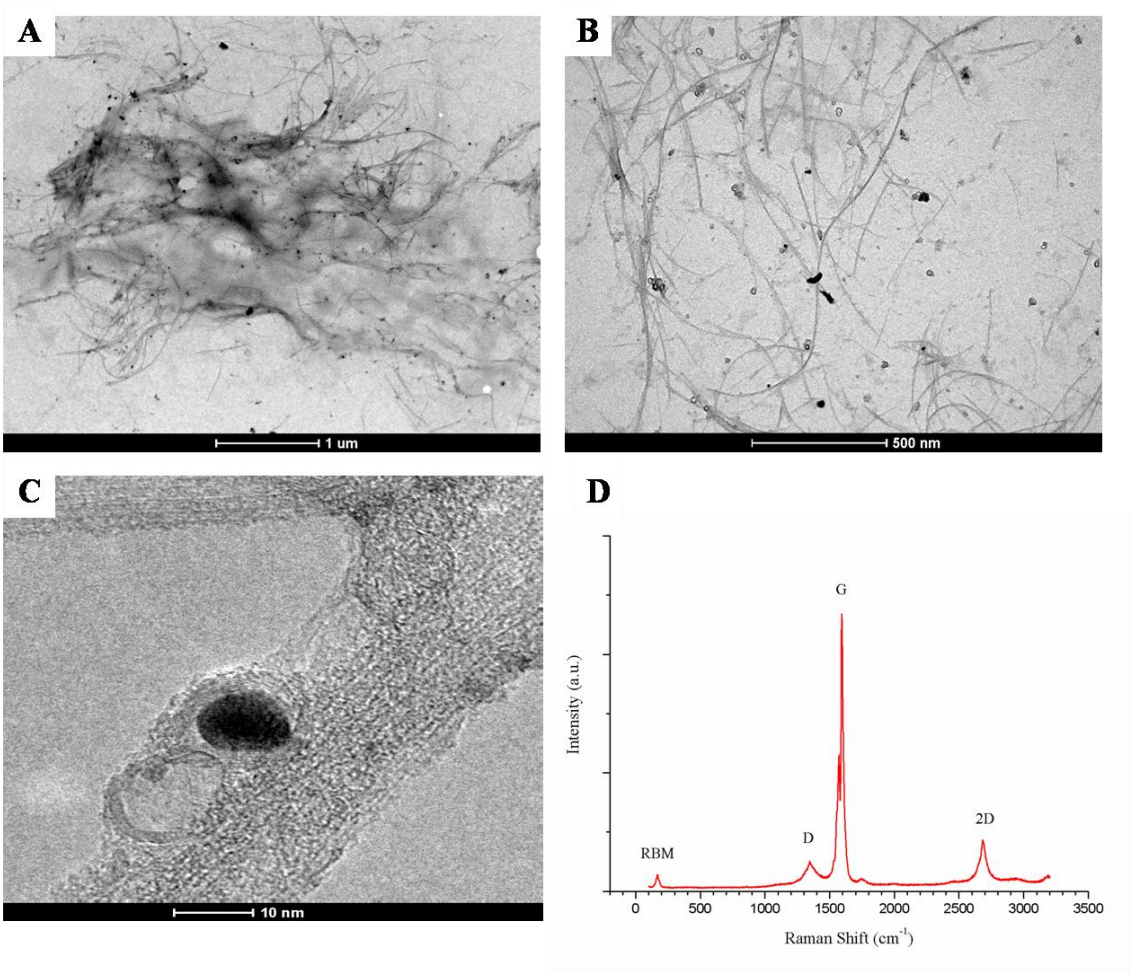
Figures

Figure 1



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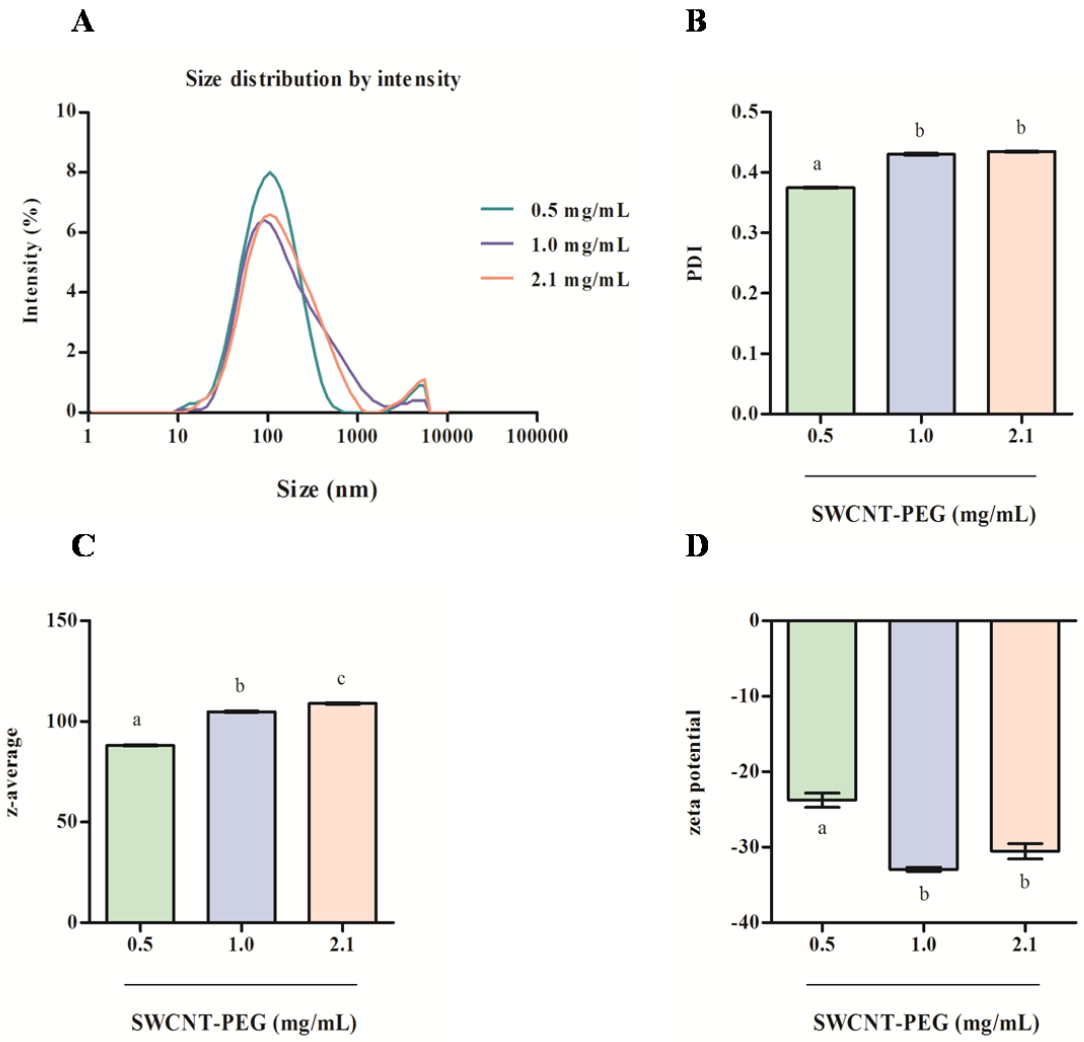
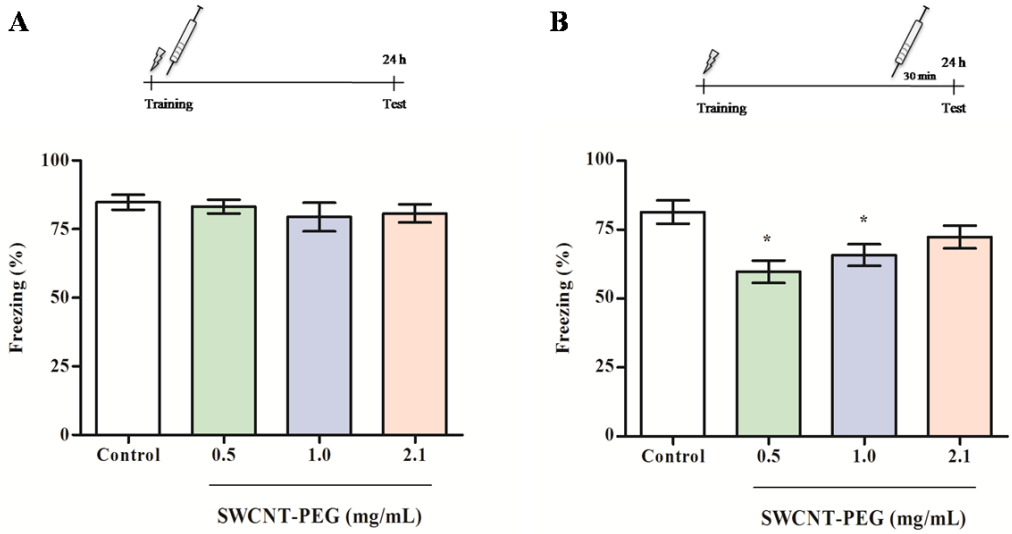

A
Size distribution by intensity
Intensity (%)
Size (nm)
0.5 mg/mL
1.0 mg/mL
2.1 mg/mL
B
PDI
SWCNT-PEG (mg/mL)
C
z-average
SWCNT-PEG (mg/mL)
D
zeta potential
SWCNT-PEG (mg/mL)

Figure 3.



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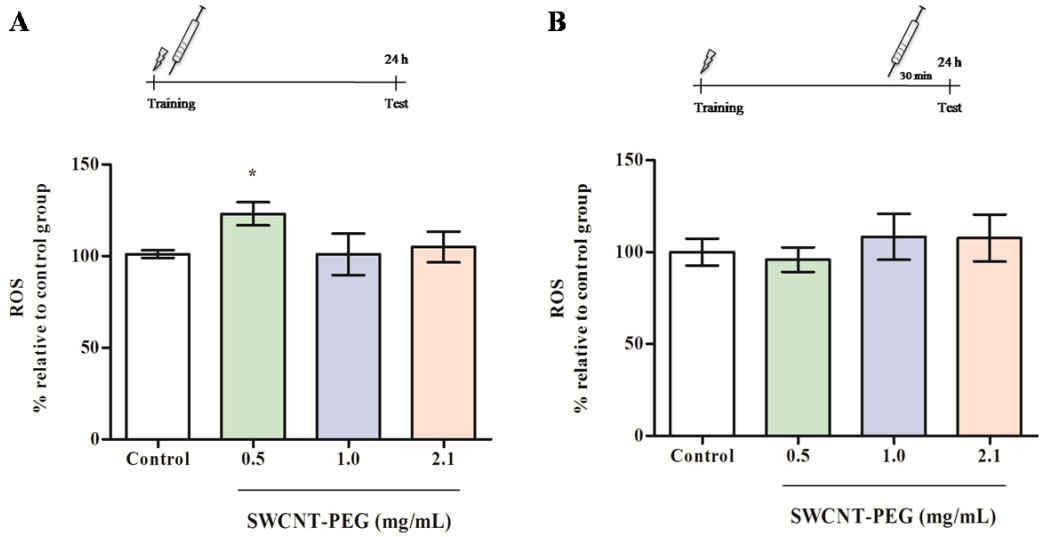


Figure 5.

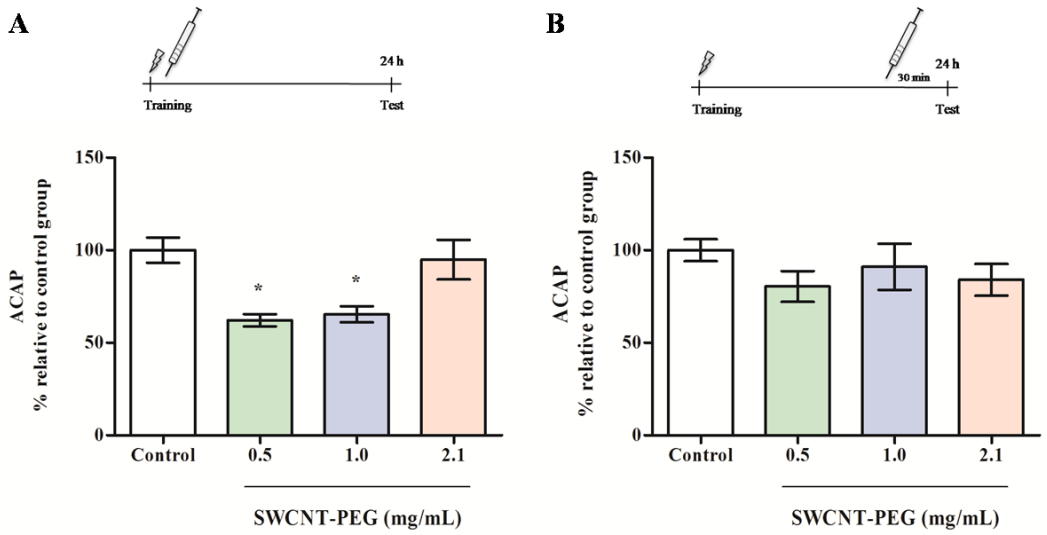


Figure 6.

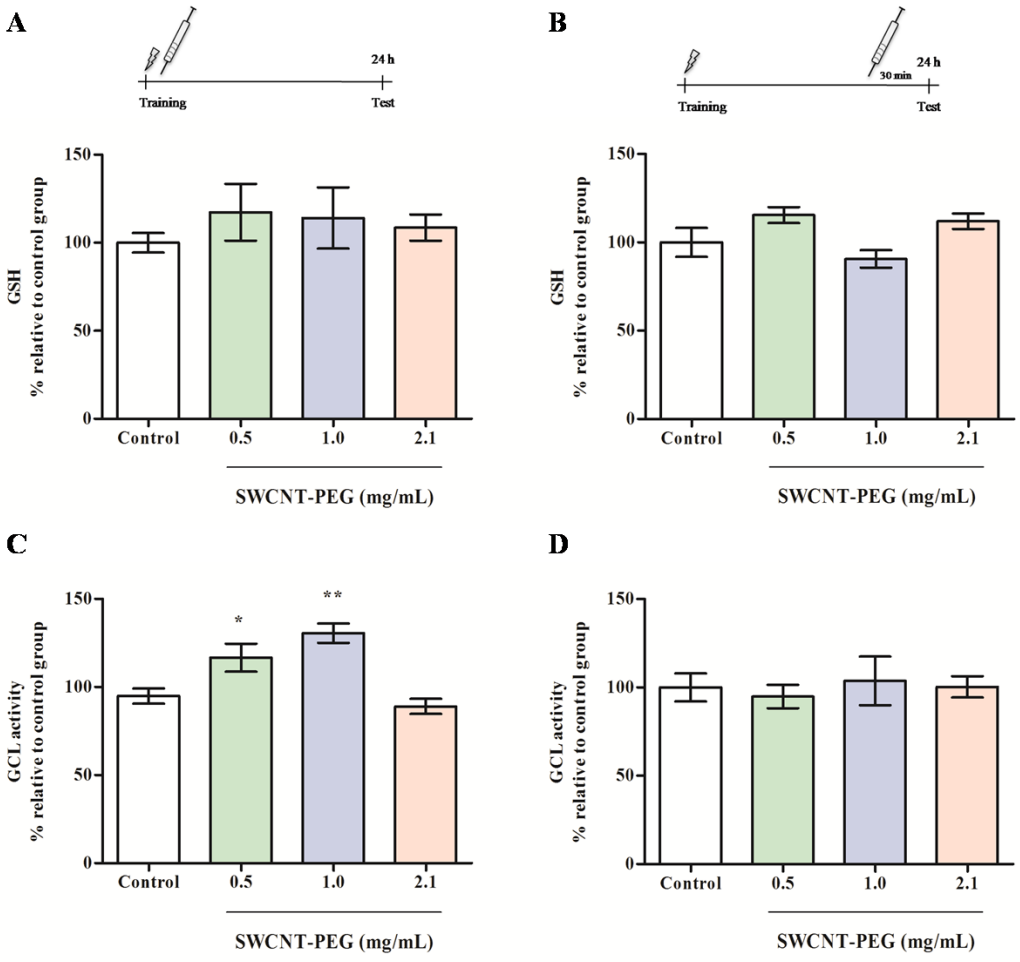
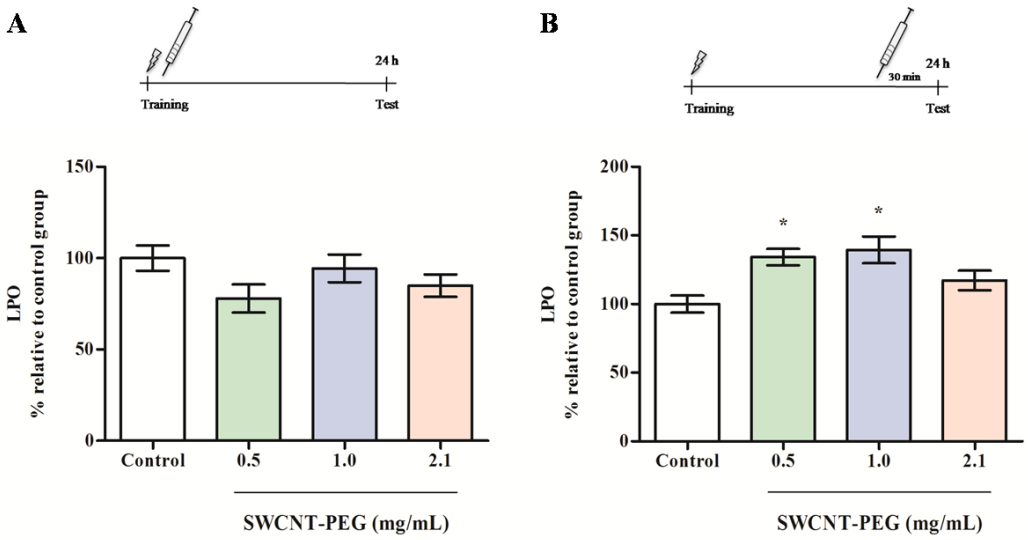


Figure 7.



Legends

Figure 1. Transmission electronic microscopy images of SWCNT-PEG dispersion before (A) and after (B) purifying and breaking the polymeric mass. High-resolution transmission electronic microscopy image (C) and Raman spectra (D)of the SWCNT-PEG after the ultrasound bath and high shear mixer treatment.

Figure 2. Intensity based particle size distribution (A), polidispersity index (PDI) (B), z-average (C) and zeta-potential of SWCNT-PEG dispersions. Different letters indicate significant difference ($P< 0.05$).

Figure 3. Effects of the SWCNT-PEG dispersions on the contextual fear memory. Infusion made immediately after training session (A) and 30 minutes before the test (B). Data represent the mean values ± SEM (n = 10-12). *Significant difference from the control group ($P< 0.05$).

Figure 4. Reactive oxygen species (ROS) generation in rat hippocampus 24 h (A) and 30 min (B) after the infusion of the SWCNT-PEG dispersions. Data represent the mean ± standard SEM (n = 4-6). *Significant difference from the control group ($P< 0.05$).

Figure 5. Effects of SWCNT-PEG dispersions on the antioxidant capacity against peroxyl radicals (ACAP) in the rat hippocampus 24 h (A) and 30 min (B) after the treatments infusion. Data represent the mean ± standard SEM (n = 4-6). *Significant difference from the control group ($P< 0.05$).

Figure 6. Glutathione (GSH) content 24 h (A) and 30 min (B) after intrahippocampal SWCNT-PEG dispersions infusion. Activity of glutamate cysteine-ligase (GCL) 24 h (C) and 30 min (D) after SWCNT-PEG dispersions infusion. Data represent mean ± standard SEM (n = 4-6). *Significant difference from the control group ($P< 0.05$). **Significant difference from the control and SWCNT-PEG 2.1 mg/mL ($P< 0.001$).

Figure 7. Effects of SWCNT-PEG dispersions on the lipid peroxidation (LPO) 24 h (A) and 30 min (B) after the treatments infusion. Data represent the mean ± standard SEM (n = 4-6). *Significant difference from the control group ($P< 0.05$).

Manuscrito 2: Biopersistence of PEGylated carbon nanotubes promotes antioxidant response 1-week after infusion in rat hippocampus

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Biopersistence of PEGylated carbon nanotubes promotes antioxidant response 1-week after infusion in rat hippocampus

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Keywords: PEGylated carbon nanotubes, biopersistence, hippocampus, antioxidant response, oxidative stress, contextual fear memory.

Abstract

Carbon nanotubes (CNT) are promising nanomaterials for diagnosis and treatment of brain disorders. However, the ability of these nanomaterials to cross cell membranes and interact with neural cells brings the need the assessment of their potential adverse effects on the nervous system. In this study, were investigated the biopersistence and neurotoxicity of single-walled carbon nanotubes (SWCNT) functionalized with polyethylene glycol (SWCNT-PEG) at 1 and 7 days after the administration in rat hippocampus. The contextual fear conditioning (CFC) task was employed to evaluate the effects of these nanomaterials on acquisition and persistence of contextual fear memory. SWCNT-PEG dispersions at 0.5, 1.0 and 2.1 mg/mL were infused in rat hippocampus 30 min before the training and 12 h after training on CFC task for the assessment of long-term memory acquisition (test performed 1 day posttraining) and persistence (test performed 7 days post training), respectively. Raman analysis of hippocampal homogenates indicates the biopersistence of SWCNT-PEG in the hippocampus 1 and 7 days after their infusion. The infusions of the nanomaterial in both experimental times had no effect on the acquisition or persistence of the contextual fear memory. SWCNT-PEG dispersions at 0.5 and 1.0 mg/mL were able to decrease the antioxidant capacity of the hippocampus 1 day after the infusion, without modify reactive oxygen species or lipid hydroperoxides levels. After 7 days of the infusion, SWCNT-PEG dispersions at all concentrations induced antioxidant defenses and reduced ROS production of the hippocampus. Such antioxidant response may constitute an adaptative response to nanomaterial biopersistence.

Introduction

Carbon nanotubes (CNT) are engineered nanomaterials with unique optical and electrical properties and large surface area that make them useful for many applications (Baughman et al., 2002), including in the biomedical field (Heister et al., 2013). CNT are classified as single-walled (SWCNT) or multi-walled (MWCNT) according with the number of graphene sheets rolled into the concentric tubes (Ijima, 1991). Both types of CNT have been extensively studied for the delivery of drugs and biomolecules (Vashist et al., 2011) and also as scaffold materials for tissue regeneration (Saito et al., 2009; Fabbro et al., 2013).

CNT are highly hydrophobic and form larger aggregates result of van der Waals interactions among the tubes (Lee & Geckeler, 2010), thus the surface functionalization is required for their biomedical applications (Heister et al., 2013). Polyethylene glycol (PEG) has been widely used as functional group for coating or covalent attachment on CNT surface because of its hydrophilic characteristic can confer high biocompatibility and dispersibility to the CNT. Moreover, it can be useful for further conjugation with bioactive molecules or fluorescent dyes (Liu et al., 2009; Bottini et al., 2011).

The ability of CNT to cross cell membranes and interact with neural cells (Cellot et al., 2011) can make them suitable for the development of drug delivery vehicles (Ren et al., 2012), gene delivery vectors (Al-Jamal et al., 2011) and biomaterials for diagnosis and treatment of brain disorders (Lee et al., 2011; Roman et al., 2012). A fundamental step towards these applications is the evaluation of CNT neurotoxicity. *In vitro* studies have demonstrated neurotoxic effects of CNT (Zhang et al., 2011; Wang et al., 2012; Meng et al. 2013). However, there are few studies on neurobehavioral changes after CNT exposure (Liu et al., 2014), so that further studies are required before using CNT *in vivo* applications.

The pathogenic potential of CNT may be related to their ability to persist in the biological systems in spite of the clearance mechanisms, which is referred as bio-durability or biopersistence (Liu et al., 2010). Although CNT are considered stable in biological environments, it has been demonstrated that certain types of CNT were enzymatically biodegradated (Allen et al., 2009; Kagan et al., 2010; Vlasova et al., 2012). A recent study of Nunes et al (2012) reported the occurrence of partial degradation of amino-functionalized MWCNT after the direct stereotactic injection in

the motor cortex, raising questions for further investigations on the factors underlying CNT *in vivo* degradation.

The aim of this study was evaluate the biopersistence and neurotoxicity of SWCNT functionalized with PEG (SWCNT-PEG) 1 and 7 days after the stereotaxic administration into the hippocampus. Raman spectroscopy was employed for the detection of SWCNT-PEG in the hippocampus based on their unique spectroscopic signatures. To evaluate the neurotoxicology, the effects of the nanomaterial on the acquisition and persistence of episodic memory were assessed by the contextual fear conditioning task and the measurements of ROS, lipid peroxidation (LPO), total antioxidant capacity against peroxyl radicals (ACAP), glutathione (GSH) content and glutamate cysteine ligase (GCL) activity were employed.

Material and methods

Preparation of SWCNT-PEG dispersions and characterization

Single-walled carbon nanotubes (SWCNT) were synthesized via the electric arc discharge method and functionalized with polyethylene glycol (PEG, Ma = 600 Da) were supplied by Sigma-Aldrich (652474, Lot MKBC 9435, ST. Louis, MO, USA). The commercial SWCNT-PEG sample was dispersed in deionized water at a high concentration (greater than 2 mg/mL). A series of steps dispersion process employing several cycles of sonication, high-shear mixing, centrifugation and ultracentrifugation were made to remove carbonaceous impurities, metal catalysts and excess unbound PEG (procedure adapted from Kalinina et al., 2011). After the entire process the concentration of the final dispersion was of 2.1 mg/mL as estimated by the intensity of light absorption at 700 nm (Shimadzu UV-Vis-NIR spectrophotometer UV-3600). The dispersions of 1.0 and 0.5 mg/mL were prepared by diluting the final dispersion with deionized water and subjecting to ultrasound bath for 10 min. The SWCNT-PEG as-produced and after dispersion protocol were characterized by transmission electron microscopy (TEM), Raman spectroscopy, energy dispersive X-ray spectroscopy (EDS), dynamic light scattering (DLS) and zeta potential measurement.

TEM (FEI Tecnai G2-Spirit 120 kV) was employed to analyze morphological characteristics of the dispersion. Raman spectroscopy (Horiba T 64000 Raman spectrometer, laser excitation wavelength - 785 nm) was used to evaluate the quality, structure and functionalization of the carbon nanotubes. In order to predict the stability

of the SWCNT dispersions the Zeta potential was determined using the electrophoretic light scattering technique on a Zetasizer (Nano-ZS ZEN3600 system - Malvern Instruments). The EDS analyses was used to show metallic impurities and the DLS (Zetasizer Nano ZS® system, Malvern Instruments) was used to determine size distribution, polydispersity index (PDI) and hydrodynamic particle diameter (z-average).

Animals handling and infusion of dispersions

In this investigation, 96 healthy adult male Wistar rats (2-3 months of age; weight 250-320 g) were used. The animals were obtained from a breeding colony of the Universidade Federal do Rio Grande (RS, Brazil) and were randomly selected and housed in polycarbonate cages (5 rats per cage) with free access to food and water. The rats were kept under standard laboratory conditions (12 h light/dark cycle and constant temperature 23 ± 1 ˚C) and frequently manipulated to avoid neophobia. All animal procedures were approved by the Ethics Committee for Animal Use of the Universidade Federal do Rio Grande (FURG; Permit Number: P029/2011).

Animals were allowed to adapt to the laboratory conditions 1 week before surgery. After this acclimation, the animals were anesthetized intraperitoneally with ketamine hydrochloride (50 mg/kg) and xylazine (4 mg/kg) and placed in a stereotaxic instrument to submit to surgery for the bilateral implant of cannula on the dorsal hippocampus. The surgical procedure was performed as described by de Aguiar et al (2013) and Parfitt et al (2012). After 48-72 h of recovery from surgery, rats were distributed randomly in groups according to the treatments (n=10-12). The dispersions of SWCNT-PEG at 0.5, 1.0 or 2.1 mg/ml or 0.9 % NaCl solution (control group) were infused using 27-gauge injection needles inserted into each guide cannula and connected by polyethylene tubing to a Hamilton microsyringe. The infusions, at a volume of 1 µl were performed in a cannula at a time.

Behavioral procedure

To study the effect of SWCNT-PEG dispersions on the acquisition and persistence of aversive memory, the animals were subjected to contextual fear conditioning (CFC) task, which was performed in chambers with internal dimensions of 28 x 26 x 23 cm aluminum walls, Plexiglas front wall and floor made of a series of a parallel stainless steel bars connected to shock scrambler deliver apparatus (shock generator, Insight Scientific Equipments, Brazil). The fear conditioning procedure was

carried out with training and test sessions, as previously described by Bekinschtein et al (2007). At training session three consecutive electric foot-shocks (1 sec duration, 0.7 mA intensity), with 10 sec of interval, were applied. The infusions of treatments were made 30 min before training (acquisition group) and 12 h after training (persistence group). Test session was performed 1 day (acquisition group) or 7 days (persistence group) after training and the freezing was quantified (absence of any movement except that required for breathing) during 5 minutes. Both training and test sessions were performed between 8:00 and 12:00 a.m. The chambers were cleaned with 70% ethanol between each set of animal. Results were expressed as the percentage of time spent in freezing in a 5 min period.

Tissue dissection and sample preparation

For analysis of oxidative stress and Raman spectroscopy the samples of tissue were removed immediately after the end of the CFC test (one group of animals twenty-four hours and the other group at seven days). All animals were killed by decapitation and their hippocampi were quickly dissected and stored at -80 ºC until use except for ROS measure, for which they were immediately homogenized (1:5 w/v) in 40 mM ice-cold Tris-HCl buffer (pH 7.4). For the analysis of antioxidant capacity against peroxyl radicals (ACAP), glutamate cysteine ligase (GCL) activity and glutathione (GSH), hippocampi were kept on ice and homogenized in buffer containing 100 mM Tris-HCl, 2 mM EDTA and 5 mM $MgCl_2$ (pH 7.75). After that, the tissue homogenates were centrifuged at 20,000 g, 4 ºC, for 20 min. For LPO analysis, hippocampi were homogenized (1:15 w/v) in 100% ice-cold methanol and centrifuged at 1000 g for 10 min at 4˚ C. For Raman spectroscopy, hippocampi were homogenized (1:2 w/v) in lyses buffer (1% SDS, 1% Triton X-100, 40mM Tris acetate, 10 mM EDTA, 10 mM DTT) using a homogenizer and sonication (1 min for each sample) as previously described by Liu et al, 2008. Shortly before the Raman spectroscopy analysis, tissue homogenates were heated at 70°C for two hours to obtain a clear lysate.

Raman spectroscopy of hippocampal homogenates

The Raman measurement of SWNT solutions was performed in the equipment Horiba T 64000 Raman spectrometer (laser excitation wavelength - 785 nm). At least three spectra were taken for each sample for averaging. Tissue samples from animals exposed to nanomaterial and positive and negative controls were analyzed. The positive control was made mixing 1 uL of SWCNT-PEG dispersion at 0.5 with 200 uL of lyses

buffer. The negative control was performed using the hippocampal homogenate from a control animal, i.e., a rat that received the infusion of 1 uL of saline solution (NaCl 0.9%). At least 50 µL of each sample was used for obtaining Raman spectrum.

Oxidative stress measurements

Sample preparation was made as previously described. After homogenization in buffer, supernatants had their total proteins content measure by Biuret method using a commercial kit (Doles, Goiânia, GO, Brazil) and the microplate absorbance reader (BioTek LX 800). The final protein concentration was adjusted to 3 mg/mL. Oxidative stress evaluation was performed by determination of reactive oxygen species (ROS) production, total antioxidant capacity against peroxyl radicals (ACAP), glutathione reduced (GSH) content, glutamate-cysteine ligase (GCL) and lipid peroxidation (LPO).

ROS production was quantified using the compound 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA, Molecular Probes Eugene, OR, USA) as previously employed for brain tissue (de Aguiar et al., 2008; Galhardi et al., 2009). In the procedure samples were placed in reaction buffer (pH 7.2) containing 200 mM KCl, 30 mM HEPES, 1 mM $MgCl_2$ and 16 µM H_2DCFDA. Using a fluorescence microplate reader (485 nm excitation/520 nm emission; Victor 2, Perkin Elmer) the formation of the oxidized fluorescent product dichlorofluorescein (DCF) was monitored with readings every 5 min for 30 min. ROS generation was calculated by integrating the fluorescent units (FU) along the time of measurement and after fitting the data to a second order polynomial function and was expressed in FU.

ACAP determination employed the quantification of ROS using H_2DCFDA (40 µM final concentration), as previously described. Hippocampus samples were treated or not with 4 mM 2,2′-azo-bis-di-hidrocloretomethylpropionamidine (ABAP, Sigma-Aldrich, St. Louis, MO, USA), a substrate that generates peroxyl radicals through thermal decomposition. DCF fluorescence was recorded by fluorescence microplate reader (485 nm excitation/520 nm emission; Victor 2, Perkin Elmer) with readings every 5 min for 30 min. The inverse of relative difference between ROS area with and without ABAP was considered as a measure of antioxidant capacity. The protocol was performed following the methodology described by Amado et al (2009).

GSH content and GCL activity were determined by the method based in the reaction of naphthalene-2,3-dicarboxyaldehyde (NDA, Molecular Probes Eugene, OR, USA) with GSH or γ-glutamyl cysteine (γ-GC) to form cyclic products that are highly

fluorescent (White et al., 2003). NDA-GSH fluorescence (472 nm excitation/528 nm emission) was measured by the fluorescence microplate reader (Victor 2, Perkin Elmer). GSH content was expressed in nM and GCL activity in nM h^{-1}.

LPO was determined by spectrophotometric assay of the ferrous oxidation/xylenol orange (FOX) modified method as previously described by Monserrat et al (2003) with adjustments in time of incubation and sample dilution according de Aguiar et al (2008). The basic reaction of this method is the oxidation of Fe(II) under acidic conditions and quantification of lipid hydroperoxides using 0.1 mM cumene hydroperoxide (CHP, Sigma-Aldrich, St. Louis, MO, USA) as a standard. CHP absorbance (550 nm) was determined using a microplate reader (BioTek LX 800) and the results were expressed by nM of CHP per gram of wet tissue.

Fluorescence-based in vitro assays

We performed the fluorescence-based *in vitro* assays to verify the potential interference of SWCNT-PEG on DCF and NDA-GSH fluorescence. For these, 1 µL of distilled water or SWCNT-PEG dispersions at 0.5, 1.0 or 2.1 mg/ml were directly added to 300 µL hippocampus extracts (protein concentration adjusted to 3 mg/mL) obtained from naïve animals and processed as introduced previously for ACAP and GSH assays. These samples were immediately subjected to ACAP and GSH biochemical measurements as prior described. Results of ACAP *in vitro* assay were expressed as ROS area with and without ABAP. Results of *in vitro* GSH assay were expressed in nM of GSH/mg of protein. Volumes of SWCNT-PEG dispersions and tissue extract used in these assays (1/300) were calculated from the higher estimated concentration of SWCNT-PEG that could remain on rat hippocampus after processing, considering the average weight of 60 µg/hippocampus and tissue dilution of 1:5 w/v.

Statistical analysis

The results of hydrodinamic size, polydispersity index and zeta-potential data were analyzed by one-way statistical analysis of variance (one-way ANOVA) followed by Tukey pos-hoc test. For the analysis of biochemical and behavioral data was used one-way ANOVA followed by Newman-Keuls multiple comparisons test. Normality and variance homogeneity were verified as ANOVA assumptions and $P<0.05$ was considered as statistically significant. The results of biochemical assays were normalized to percentage of the control group.

Results and discussion

The physical dimensions and surface properties are critical factors underlying CNT biological effects (Johnston et al., 2010), thus, a detailed characterization is indispensable to understand the nanomaterial interactions in a biological system. In this work, the characterization through transmission electron microscopy (TEM) showed that SWCNT-PEG were functionalized in the form of bundles and even after the dispersion protocol was not possible separate them into individual tubes (Fig. 1A). The length of the bundles was heterogeneous, with measures mostly between 600 nm and 800 nm. We can also observed metallic catalyst residues in the form of nanoparticles coated with graphitic multilayer (nano-onions) that were functionalized and linked covalently to the beams, preventing their removal by the centrifugation steps. Some of these nano-onions were empty after dispersion protocol (Fig. 1B, inset). EDS analysis showed that metallic impurities mainly composed of nickel and yttrium remained in SWCNT-PEG final dispersion. These transition metals are two catalysts commonly used in the synthesis of nanotubes by electric arc (Laplaze et al., 2002) that probably remained in the SWCNT-PEG sample after the purification in a stable and non-bioavailable (encapsulated) form, as indicated by the presence of nano-onions.

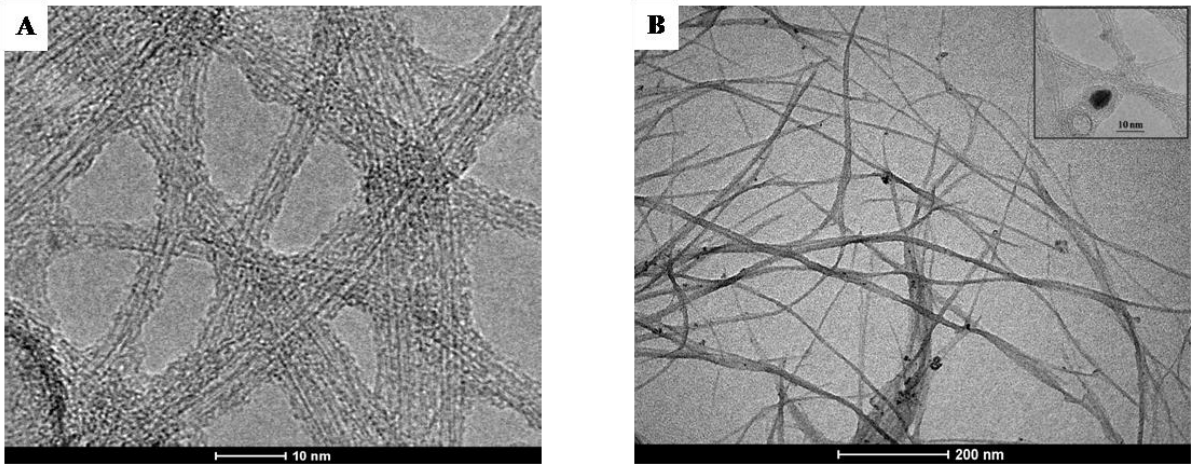

Fig. 1. TEM images of SWCNT-PEG after the dispersion protocol. (A) High-resolution TEM of SWCNT-PEG dispersion shows functionalized bundles containing, in average, from 5-10 tubes. (B) Low-resolution TEM image of SWCNT-PEG final dispersion shows the fibers and metallic nanoparticles (inset: high resolution TEM of metallic nanoparticle coated with graphitic multilayer).

The hydrodynamic size and zeta potential of SWCNT-PEG dispersed in water are shown in Table 1. The hydrodynamic size increases with the concentration of

SWCNT-PEG in the dispersions, and the dispersion at 2.1 mg/mL presented the higher size of particle agglomerates. Zeta potential values of SWCNT-PEG dispersions at 0.5, 1.0 and 2.1 mg/mL were -23, -32, and -30 mV, respectively, which may indicate remaining carboxyl acid groups on CNT surface after the oxidation process used for SWCNT functionalization with PEG (Zhao et al., 2005). It was proposed that carboxylic groups and defects on the graphitic surface are the primary sites for interaction with the oxidative agents and consequent CNT degradation (Russier et al., 2011), even for PEGylated CNT (Allen et al., 2009; Liu et al., 2010). However, SWCNT-PEG used in this work remained in the hippocampus up to 7 days after infusion, as indicated by Raman spectroscopy analysis (Fig. 2). The physicochemical properties of SWCNT-PEG that may have contributed to spare them from biodegradation were not elucidated in this study.

Table 1

Zeta potential and hydrodynamic size of SWCNT-PEG dispersions.

SWCNT-PEG dispersion	Zeta potential (mV)	Hydrodynamic size (nm)
0.5 mg/mL	-23.77 ± 0.95	88.05 ± 0.27
1.0 mg/mL	-32.93 ± 0.29*	104.8 ± 0.32*
2.1 mg/mL	-30.53 ± 1.02*	108.9 ± 0,32*$^{\#}$

Values are expressed as mean ± SEM (n=3). *$p<0.05$ vs SWCNT-PEG 0.5 mg/mL; $^{\#}p<0.05$ vs SWCNT-PEG 1.0 mg/mL (one-way ANOVA followed by Tukey pos hoc test).

Raman spectroscopy is a molecular vibrational spectroscopy that provides important information about CNT structure and purity (Dresselhaus et al., 2003; Dillon et al., 2004). The specificity of this technique allows the accurate detection of CNT in complex biological systems and has been widely used to probe the biodistribution of SWCNT in various organs of mice (Liu et al., 2008; Zavaleta et al., 2008; Ingle et al., 2013). Moreover, Raman spectroscopy can detect defects in SWCNT structure after the oxidative modification by myeloperoxidase *in vivo* (Kagan et al. 2010).

Here we employed Raman spectroscopy analysis to verify how the CNT are functionalized and to detect the SWCNT-PEG in the hippocampus 1 and 7 days after

the direct administration in the hippocampus. The vibrational modes present in Raman spectra of SWCNT-PEG were the radial breathing mode (RBM), D band and G band (Fig. 2A). D and the G band are characteristic of graphitic carbon structure of SWCNT. The D band is associated with the defects and other impurities in the graphitic structure of the CNT (Dillon et al., 2004), and G band correspond to the stretching modes of the carbon-carbon bonds in the graphene planar structure (Dresselhaus et al., 2003).

In the analysis of Raman spectrum of SWNT-PEG, we found a well defined and intense G band, which is not expected in functionalized samples. This observation confirms that the functionalization of SWCNT occurs in the bundles rather than in individual CNT. The detection of SWCNT-PEG in the hippocampal homogenates was determined based on the RBM, a Raman mode unique for SWCNT. The position and shape of RBM, generally between 140 and 220 cm^{-1}, depends strongly on the exciting laser wavelength and can be affected by the intertube interaction in the bundles (Kuzmany et al., 2001). The Raman spectra of SWCNT-PEG dispersions diluted in order of 10 were obtained to establish the detection curve of the nanomaterial (Fig. 2B).

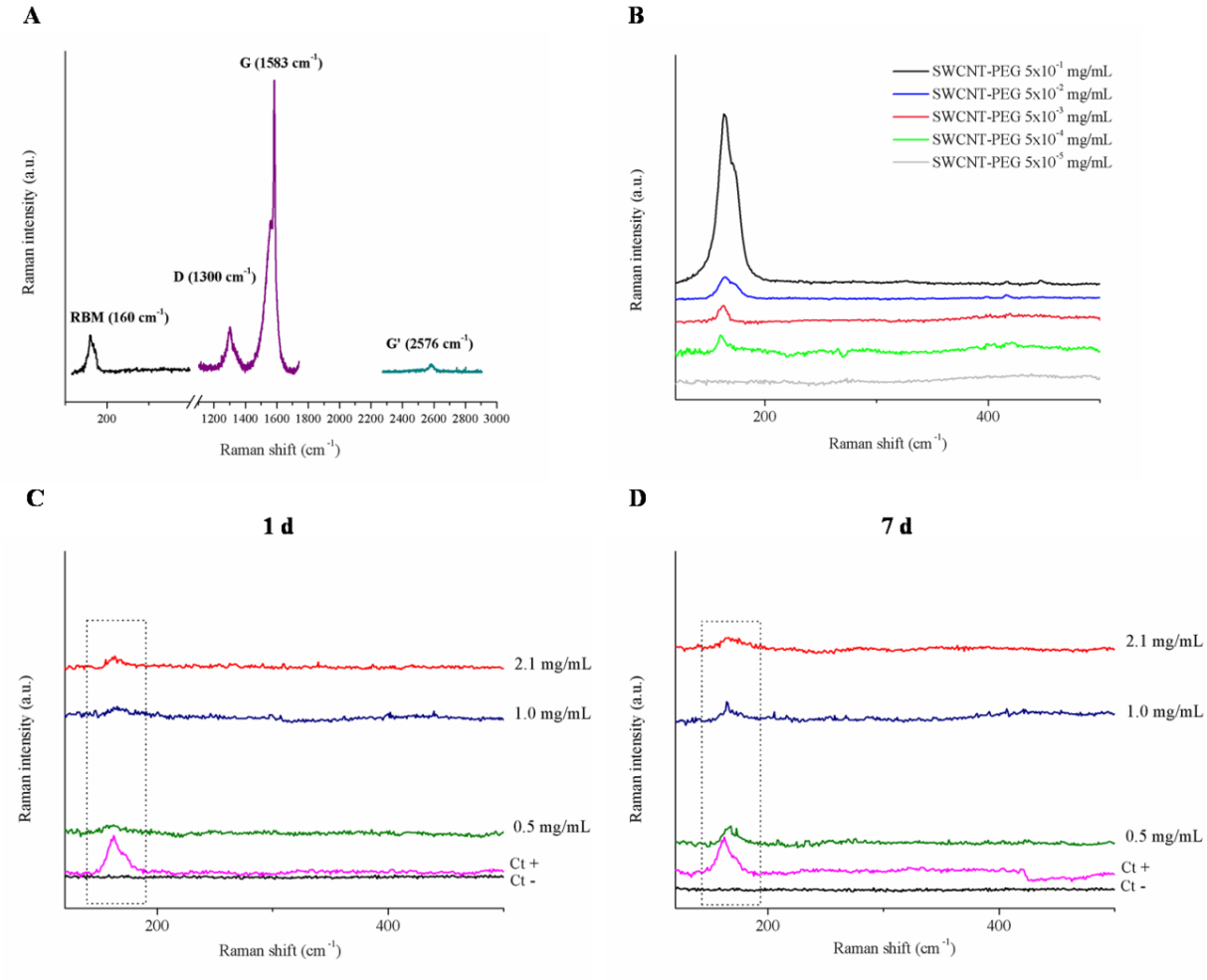

Fig. 2. Raman spectra of SWCNT-PEG in the dispersion (A and B) and in hippocampal homogenates (C and D). The RBM was used to perform the detection curve (B) and to detect SWCNT-PEG in hippocampal homogenates 1 day (C) and 7 days (D) after their infusion. Dotted squares indicate the RBM region. Ct+: positive control; Ct-: negative control.

Raman analysis of tissue homogenates indicates that at least part of SWCNT-PEG remained unchanged up to 7 days after their infusion in the hippocampus (Fig. 2C and D). If by one hand the biodegradation of CNT can facilitate the elimination and reduce toxicity of these nanomaterials (Kagan et al., 2010), on the other hand it can generate degradation products, as oxidized aromatic hydrocarbons (Allen et al., 2009), that could cause unpredicted toxicity. Thus, the consequences of the biopersistence of CNT in organs and tissues of living organisms should be evaluated carefully. Moreover, we should consider that more time may be required for the complete degradation of SWCNT-PEG in the hippocampus. It was reported that pulmonary oxidative biodegradation of SWCNT can take several weeks (Shvedova et al., 2012), whereas the degradation of amino functionalized MWCNT in mice brain can start within 2 days postinjection (Nunes et al., 2012). Such differences may reflect the interaction of many factors in the biodegradation process, as the enzymatic profile of tissue and the type of surface functionalization of CNT.

In this work, the infusions of SWCNT-PEG dispersions in rat hippocampus were performed 30 min before the training and 12 h post training in the CFC task. The infusions made before the training session allowed us to evaluate if the nanomaterial affects the acquisition, i.e., the first stage of memory processing in which is established an association between the context and the shock (Abel & Lattal, 2001). While the infusions performed 12 h post training aimed to evaluate if the SWCNT-PEG can impair cellular and molecular late events that occur in the rat hippocampus 12 h post training and are required for fear memory persistence 7 days after conditioning (Bekinschtein et al. 2007). Our results showed that SWCNT-PEG dispersions infusions had no effects in the acquisition and persistence of the contextual fear memory (Fig. 3), as indicated by similar time spent in freezing between the treated animals and the control group.

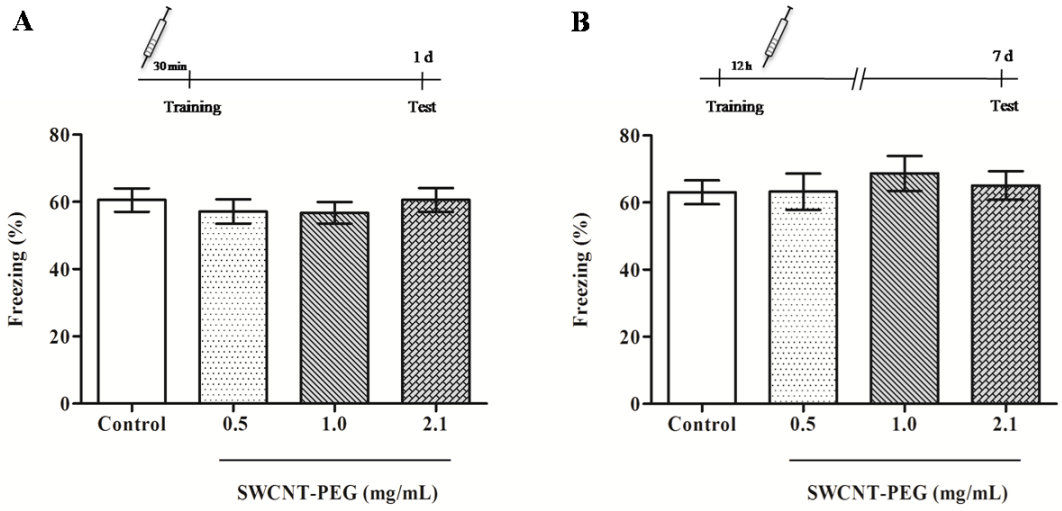

Fig. 3. Effect of SWCNT-PEG dispersions in the contextual fear conditioning for animals infused 30 min before training and tested after 24 h (A) and for infusions performed 12 h after training and test at 7 days (B). Schematic of the procedure used in the experiment are shown above the graphs. Values are expressed as mean ± SEM, n = 10-12. There are no significant differences between the groups in both experimental periods.

Considering the role of oxidative stress as an important mechanism underlying the neurotoxicity of CNT (Wang et al., 2012; Liu et al., 2014) and the high vulnerability of neurons of dorsal hippocampus to oxidative stress (Wang et al., 2005), the absence of cognitive deficits found in this study were in agreement with our results from ROS production and LPO. We found a decreased ROS production in the hippocampus 7 days after SWCNT-PEG dispersions infusion, without any change at 1 day (Fig. 4). Lipid hydroperoxides levels were unaltered in both experimental times (Fig. 5).

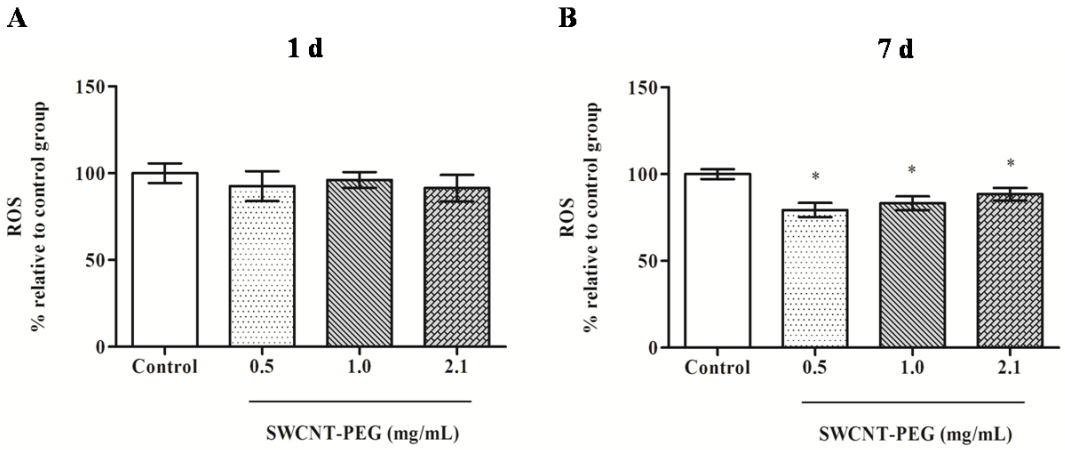

Fig. 4. Effect of SWCNT-PEG dispersions on ROS production in the hippocampus of rats 1 day (A) and 7 days (B) after the infusion. Values are expressed as mean ± SEM, n = 4-6. *p<0.05 vs control group (one-way ANOVA followed by Newman-Keuls post hoc test).

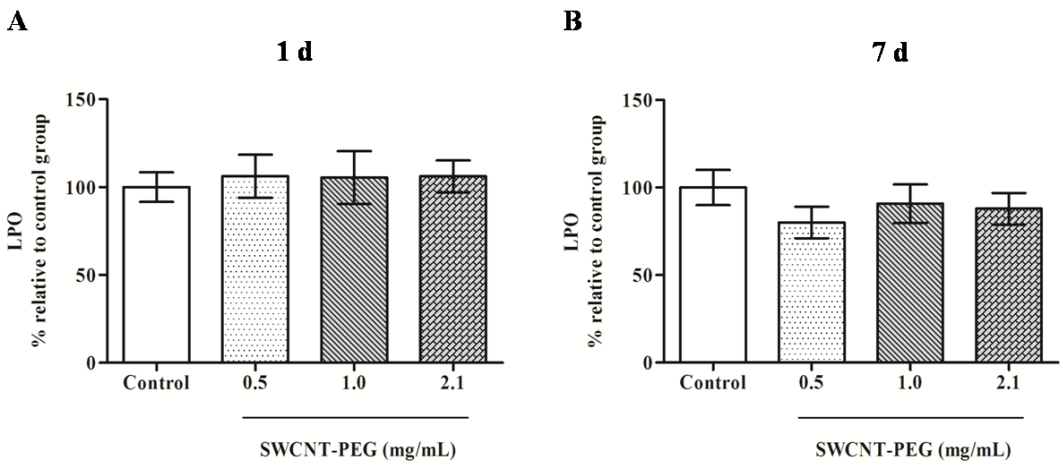

Fig. 5. Effect of SWCNT-PEG dispersions on LPO levels in the hippocampus of rats 1 day (A) and 7 days (B) after the infusion.Values are expressed as mean ± SEM, n = 4-6. There are no significant differences between the groups in both experimental periods.

The decrease in ROS production was previously observed in lungs after double-walled CNT instillation (Crouzier et al., 2010). Such effect was attributed to the ROS scavenger capability of CNT and based in the assumption from Fenoglio et al (2006) that certain ROS types may be readily linked at the surface of CNT by mechanisms similar to the grafting of organic functionalities. However, the decrease in ROS production found in this work may not to be attributed to the scavenging action of SWCNT-PEG, since the *in vitro* assays of ROS and ACAP did not shown any antioxidant activity (Table 2). Moreover, the decrease in the ACAP of the hippocampus was observed 1 day after the infusion of dispersions, without changes in ROS production. Therefore, we propose that the biopersistence of SWCNT-PEG in the hippocampus 7 days after their infusion could have induced the antioxidant defenses, resulting in the decreased ROS levels.

The determination of the integrated antioxidant response may be useful since it provides a general scenario of the oxidative status of the tissue (Amado et al., 2009). Here we found a time-dependent change in the ACAP after SWCNT-PEG infusion in the hippocampus (Fig. 6). The ACAP was lowered 1 day after the infusion of dispersions at 0.5 and 1.0 mg/mL (Fig. 6A), suggesting the mobilization of the antioxidant defenses in the initial period postinjection, which may have contributed to prevent oxidative damage, since the biomarkers of oxidative stress, i.e. ROS and LPO, were unchanged.

Assuming that cellular uptake of CNT could be facilitated by individual CNT or smaller aggregates, as already proposed (Kam et al., 2004; Jin et al., 2009; Johnston et al., 2010), the small size of SWCNT-PEG agglomerates at 0.5 and 1.0 mg/mL may explain the decrease in ACAP. However, distinct observation was seen 7 days after infusion, as presented below (Figs. 6 and 7), suggesting that small size SWCNT-PEG agglomerates were critical for the initial biological response, while the dispersion with the higher agglomerate size required a longer time to spread and interact with hippocampal cells. The biopersistence of SWCNT-PEG over time may have an important effect on the antioxidant response that culminates in the increased ACAP and GSH content (Fig. 6B).

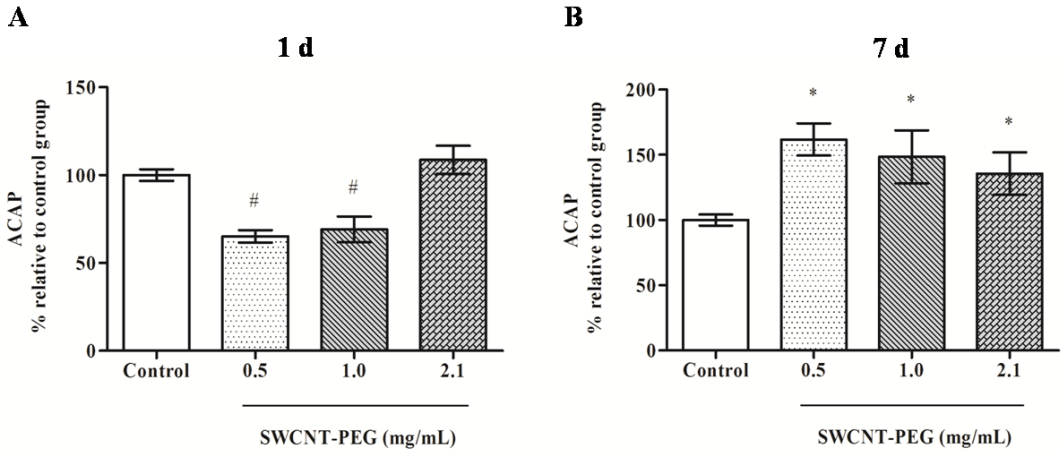

Fig. 6. Effect of SWCNT-PEG dispersions on ACAP of the hippocampus of rats 1 day (A) and 7 days (B) after the infusion. Values are expressed as mean ± SEM, n = 4-6. $^{\#}p<0.05$ vs SWCNT-PEG 2.1 mg/mL and vs control group; $^{*}p<0.05$ vs control group (one-way ANOVA followed by Newman-Keuls post hoc test).

The brain contains multiple antioxidant defenses, among which the reduced glutathione (GSH) is especially important (Halliwell, 2001). This ubiquitous tripeptide is an effective scavenger of ROS and is used to maintain the thiol/disulfide redox state of proteins and other antioxidants in their reduced and functional forms (Jones, 2006). A variety of different compounds increase GSH levels in cells by increasing the activity of the glutamate cysteine ligase (GCL), the enzyme that catalyzes the first and rate-limiting step in the synthesis of GSH (Maher, 2006). The gene expression of GCL is well regulated in order to maintain intracellular levels of GSH. The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) controls the basal and inducible

expression of genes encoding the catalytic and regulatory chains of GCL. In response to oxidative stress, Nrf2 translocates to the nucleus and bind to antioxidant response element, inducing the transcription of GCL and others cytoprotective enzymes (Kobayashi & Yamamoto, 2005).

The indirect antioxidant activity of a carbon nanomaterial was demonstrated by the pretreatment of cells with a polyhydroxylated fullerene derivative that was able to restore of the Nrf2 expression level after a neurotoxic insult (Cai et al., 2008). The increased expression of Nrf2 was also reported in the brains of zebrafish after systemic exposure to SWCNT (da Rocha et al., 2013). These studies demonstrated the ability of carbon nanomaterials to induce antioxidant defenses and prevent their potential damage to the neural tissue. In this work, there was an increase in GSH content 7 days after the infusion of SWCNT-PEG dispersions (Fig. 7B), which may have contributed to the higher ACAP. Although no raise in GCL activity was observed at 7 days (Fig. 7 D), it may have occurred previously to this period.

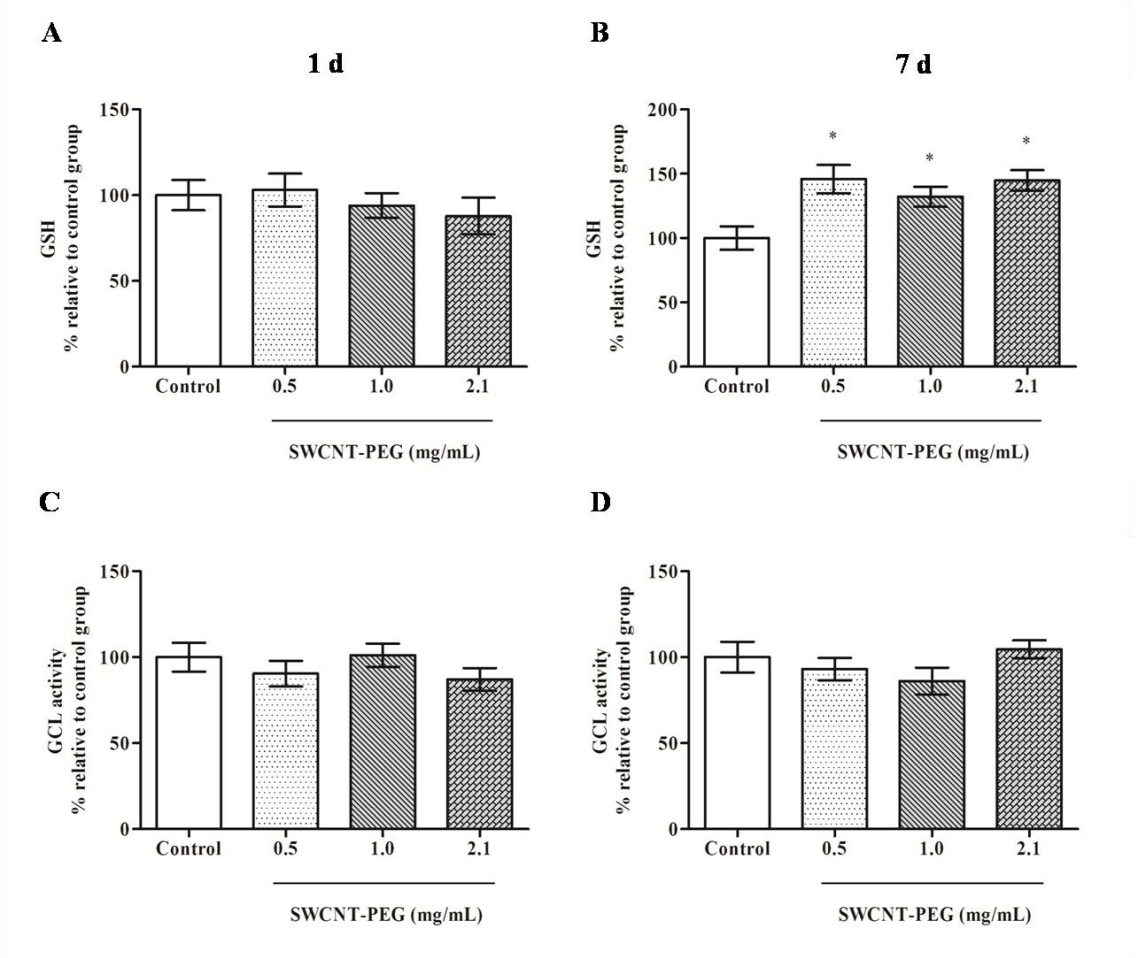

Fig. 7. Effect of SWCNT-PEG dispersions on GSH content (A and B) and GCL activity (C and D) 1 day (A and C) and 7 days (B and D) after their infusion in the hippocampus. Values are expressed as mean ± SEM, n = 4-6. *p<0.05 vs control group (one-way ANOVA followed by Newman-Keuls post hoc test).

We suggest that SWCNT-PEG exposure can induce an antioxidant response in the hippocampus that may confer resistance or adaptation to the initial insult. Furthermore, higher antioxidant levels may have contributed to the biopersistence of SWCNT-PEG in the hippocampus, since the addition of the antioxidants ascorbic acid and glutathione suppressed the *in vitro* biodegradation of oxidized-SWCNT induced by hypochlorite and myeloperoxidase (Kotchey et al., 2013).

Based on the evidence that the fluorescence of DCF can be partially quenched by SWCNT (Ren and Zhong, 2010), we performed the fluorescence-based *in vitro* assays to ensure that the changes in ROS, ACAP and GSH found 7 days after the infusion of SWCNT-PEG were resulting from the biological response to nanomaterial exposure. Results from fluorescence-based *in vitro* assays are summarized in Table 1. SWCNT-PEG dispersions did not quenching the DCF-induced fluorescence, both in presence or not of the peroxyl radicals generator ABAP. Moreover, we can discard any interference of the SWCNT-PEG on GSH content or GCL activity determination, since no interference of SWCNT-PEG was observed in NDA-GSH fluorescence generation. These results allow the interpretation of the aforementioned measurements from *in vivo* biochemical assays as reliable biological responses.

Table 2

SWCNT-PEG effects on fluorescence-based *in vitro* assays.

SWCNT-PEG dispersion	ROS area without ABAP	ROS area with ABAP	GSH (nM/mg protein)
0 mg/mL	207000 ± 13350	1176000 ± 18240	224.0 ± 22.01
0.5 mg/mL	227900 ± 16530	1139000 ± 38350	236.1 ± 25.05
1.0 mg/mL	218600 ± 17318	1238000 ± 73450	233.2 ± 28.96
2.1 mg/mL	229100 ± 20250	1134000 ± 78140	222.9 ± 24.94

Values are expressed as mean ± SEM (n= 4). There are no significant differences between SWCNT-PEG dispersions and water control.

Conclusions

The biopersistence of functionalized SWCNT in nervous system and its neurobehavioral effects are important issues to be studied before their biomedical applications. In this study, SWCNT-PEG infused in the hippocampus were detected 7 days after their infusion. The SWCNT-PEG dispersions infusions did not impair the acquisition and persistence of contextual fear memory, but was able to induce the antioxidant defenses, possibly after an initial pro-oxidant effect. The antioxidant response observed in this study may constitute an adaptative response to the nanomaterial biopersistence, which was associated to high glutathione content that may provide protection against the initial oxidative damage and prevent the biodegradation of the nanomaterial in the tissue. Further studies on the gene expression and cell signaling pathways are needed to elucidate the potential mechanisms that may confer adaptation to SWCNT-PEG biopersistence.

Acknowledgments

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Conflict of Interest

The authors declare there are no conflicts of interest.

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Considerações finais

Os principais resultados encontrados neste trabalho estão representados no esquema abaixo.

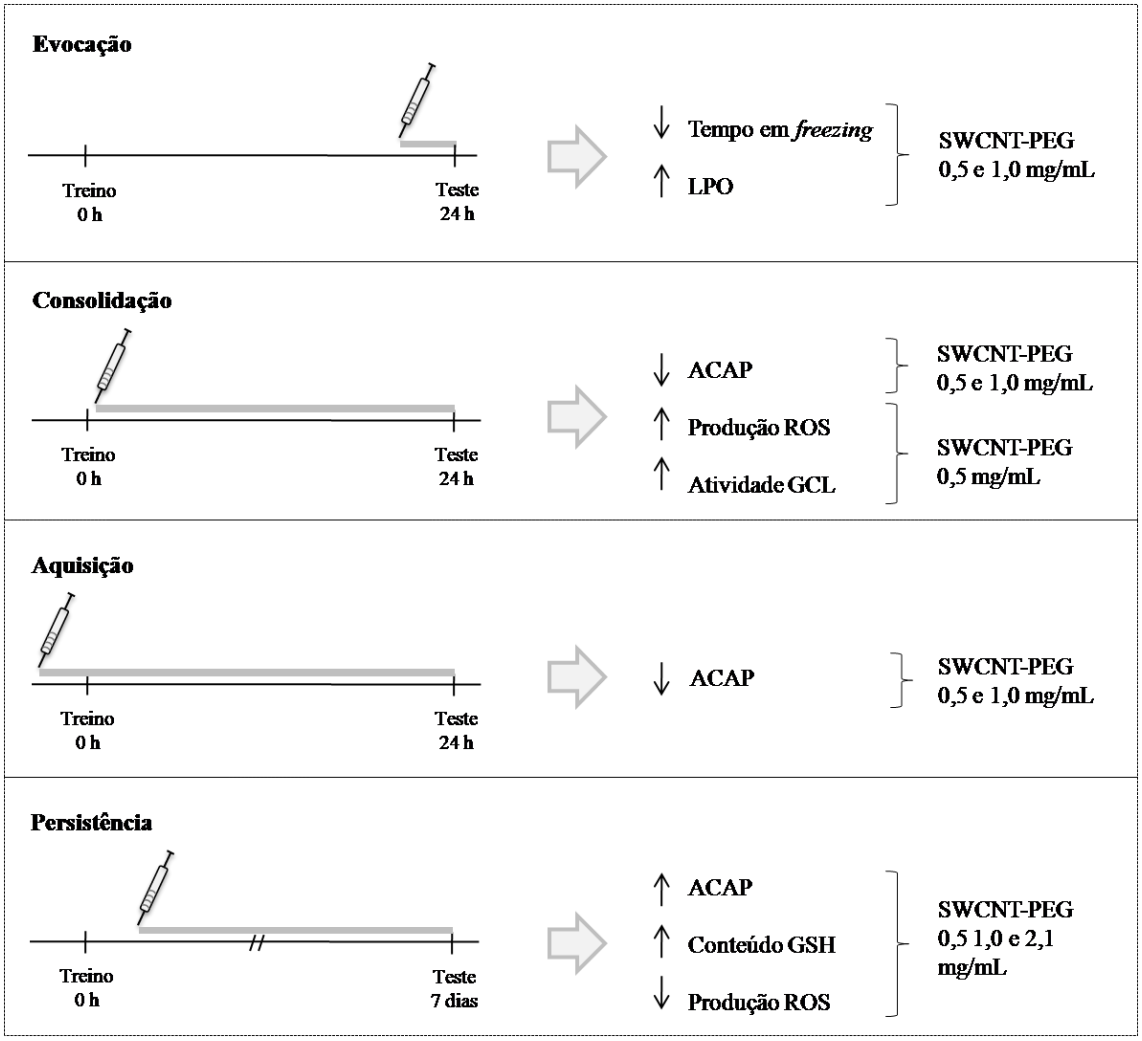

Figura 10. Resumo dos resultados. As barras cinza acima da linha do tempo indicam a duração da exposição aos SWCNT-PEG.

No presente trabalho foi demonstrado que os SWCNT-PEG podem causar déficit na evocação da memória aversiva de longa duração e produzir alterações em parâmetros de estresse oxidativo no hipocampo de ratos, sendo evidenciada a variação temporal na resposta biológica a este nanomaterial. A ação pró-oxidante dos SWCNT-PEG no hipocampo dos ratos foi detectada nas primeiras horas após a exposição. O dano oxidativo observado 30 min após a infusão das dispersões foi sucedido pela diminuição da capacidade antioxidante do hipocampo 24 h depois. Estas alterações

ocorreram nas concentrações de 0,5 e 1,0 mg/mL, o que levantou a hipótese de diferenças no estado de aglomeração dependentes da concentração dos SWCNT-PEG nas dispersões. De fato, análise da distribuição do tamanho das partículas evidenciou um maior diâmetro hidrodinâmico médio das partículas de SWCNT-PEG na dispersão de 2,1 mg/mL, o que pode ter limitado sua interação com alvos celulares e a ocorrência de toxicidade, conforme já proposto por alguns autores para outros tipos de CNT (Hirano et al., 2008; Coccini et al., 2009).

A indução de estresse oxidativo pelos SWCNT-PEG infundidos no hipocampo previamente ao teste da tarefa do MCC provavelmente esteja relacionada ao prejuízo na evocação da memória aversiva. O hipocampo é uma das estruturas do SNC mais sensíveis aos efeitos do estresse oxidativo (Wang & Michaelis, 2012), especialmente a subregião CA1, em que há uma alta demanda por ROS e RNS como moléculas de sinalização e uma produção de ATP relativamente baixa, conferindo aos neurônios desta subregião uma alta sensibilidade aos estímulos pró-oxidantes (Wang et al., 2005). A exposição a diferentes nanopartículas foi relacionada à indução de estresse oxidativo no hipocampo e alterações no aprendizado em roedores (An et al., 2012; Hardas et al., 2013; Wu et al., 2013; Ze et al., 2014). Em relação aos CNT, um estudo recente demonstrou que a administração sistêmica e repetida de SWCNT causou estresse oxidativo e inflamação no cérebro, com alterações histopatológicas no hipocampo e comprometimento da memória espacial nos animais expostos (Liu et al., 2014).

A infusão intrahipocampal dos SWCNT-PEG culminou, na diminuição da ACAP do hipocampo após 24 h de exposição. Considerando que não houve alteração no conteúdo de GSH, sugere-se que outros antioxidantes de baixo peso molecular estejam sendo oxidados e contribuindo para a proteção do tecido contra o insulto oxidativo inicial, especialmente o ácido ascórbico que está presente em altas concentrações no SNC (Rice, 2000). Este cenário de redução da ACAP foi acompanhado pelo aumento na produção de ROS e na atividade da GCL no grupo infundido com a dispersão de SWCNT-PEG de 0,5 mg/mL imediatamente após o treino na tarefa do MCC, sugerindo que o estado redox do tecido foi lentamente restabelecido neste grupo experimental, embora não houve prejuízo na consolidação da memória aversiva dos animais expostos.

Decorridos sete dias da infusão das dispersões de SWCNT-PEG, observou-se o aumento da ACAP e do conteúdo da GSH no hipocampo. Tais respostas foram observadas em todas as concentrações do nanomaterial, inclusive na que apresentou maior tamanho médio de partículas na dispersão. Este resultado sugere que ao longo do

tempo os SWCNT-PEG aumentaram sua dispersabilidade no hipocampo, possivelmente permitindo sua interação com os constituintes celulares e a geração da resposta biológica. A indução das defesas antioxidantes pode constituir um importante mecanismo de adaptação do hipocampo à biopersistência dos SWCNT-PEG, visto que os mesmos foram detectados no hipocampo 7 dias após a infusão, contrariando evidências que indicam a biodegradação dos CNT poucos dias após sua infusão no córtex cerebral (Nunes et al., 2012).

O aumento do conteúdo de GSH, cuja manutenção é essencial para prevenir o dano oxidativo ao cérebro (Dringen, 2000), pode ter sido decorrente da indução na expressão da enzima limitante na sua síntese, a GCL. No entanto, a atividade desta enzima não estava aumentada aos 7 dias, o que não descarta a possibilidade do aumento na sua expressão/atividade ter ocorrido previamente a este tempo experimental. A modulação de sistemas antioxidantes envolve diversas moléculas sinalizadoras, dentre as quais se destaca o fator nuclear eritroide 2 relacionado ao fator 2 (Nrf2), importante fator de transcrição que em condições de estresse oxidativo é translocado ao núcleo celular, ligando-se ao elemento de resposta antioxidante (ARE) e regulando expressão de enzimas de detoxificação e reposta antioxidante, dentre as quais a GCL e outras enzimas relacionadas ao sistema da GSH (Nguyen et al., 2009).

A ativação da via Nrf2-ARE também foi relacionada ao aumento na expressão da enzima heme oxigenase-1 (HO-1) em células expostas à CNT (Brown et al., 2010) e a nanopartículas metálicas (Kang et al., 2012; Aueviriyavit et al., 2014). A HO-1 é uma enzima essencial para o catabolismo do grupo heme, cujos produtos finais, monóxido de carbono, Fe^{2+} e bilirrubina, são hábeis em neutralizar ROS (Ryter et al., 2006; Paine et al., 2010). A indução de HO-1 em neurônios ou células endoteliais vasculares cerebrais constitui um importante mecanismo de proteção contra neurotoxinas ou processos inflamatórios no SNC (Syapin, 2008; Innamorato et al., 2009).

Estas evidências indicam que outros mediadores, além da GSH, podem estar envolvidos na neuroproteção e adaptação à biopersistência dos SWCNT-PEG no hipocampo. A análise da expressão de do NrF2 e da HO-1 poderá elucidar sua participação na resposta adaptativa aos SWCNT-PEG. Ademais, a análise por microscopia óptica e eletrônica e estudos com maior duração poderão contribuir na determinação da localização celular e subcelular dos SWCNT-PEG e quão extensa sua biopersistência no hipocampo.

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