PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL
FACULDADE DE BIOCIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

MODULAÇÃO DA NEUROTRANSMISSÃO COLINÉRGICA COMO RESPOSTA AOS EFEITOS CAUSADOS PELA EXPOSIÇÃO AO NANOCOMPOSTO DE CARBONO FULERENO C60 UTILIZANDO ZEBRAFISH (*Danio rerio*) COMO MODELO DE ESTUDO

Gonzalo Ogliari Dal Forno

Orientador
Prof. Dr. Maurício Reis Bogo

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Dissertação apresentada como requisito parcial para a obtenção do grau de Mestre pelo Programa de Pós-Graduação em Biologia Celular e Molecular, da Faculdade de Biociências da Pontifícia Universidade Católica do Rio Grande do Sul.

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Porto Alegre
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RESUMO

Nanocompostos derivados de átomos de carbono têm sido foco de interesse para aplicações em vários campos industriais, tais como engenharia eletrônica, produtos farmacêuticos, dispositivos médicos, cosméticos, embalagens de alimentos, entre outros, desde seu descobrimento em 1985. O fulereno C_{60} é um nanocomposto com 60 átomos de carbono que tem sido alvo de inúmeros estudos por suas propriedades como a capacidade de armazenar átomos em seu interior e aceitar diversas modificações estruturais. A importância de entendermos todos os possíveis efeitos de uma exposição aos fulerenos é muito grande, já que a sua produção e sua utilização estão crescendo a cada dia aumentando a exposição do meio ambiente a este composto. O sistema colinérgico tem como principal neurotransmissor a acetilcolina (ACh). A acetilcolinesterase (AChE, E.C.3.1.1.7) é uma importante enzima regulatória que controla a transmissão de impulsos nervosos através da sinapse colinérgica pela hidrólise da ACh. Os níveis de AChE são controlados pela interação da ACh com seus receptores, sendo que quando a interação é acentuada, aumentam os níveis de AChE Portanto, a AChE pode ser usada como um marcador da função colinérgica. Neste estudo nosso objetivo foi verificar se injeções intraperitoneais de fulereno C_{60} nas doses de 7,5; 15 e 30 mg/kg e nos tempos de 6h, 12h e 24h de exposição causaria alguma alteração na modulação do sistema colinérgico. Observamos que a dose de 30 mg/kg, no tempo de exposição de 24h, apresentou um aumento de 84% na atividade enzimática quando comparado com o grupo controle-veículo. Estes resultados sugerem um possível efeito neurotóxico, embora estudos adicionais devam ser realizados para estender estes achados.

Palavras chave: fulereno C_{60}, zebrafish, Danio rerio, nanotoxicidade

ABSTRACT

Nanocomposites derived from carbon atoms have been the focus of interest in various industrial fields such as electronic engineering, pharmaceuticals, medical devices, cosmetics, food packaging and other, since its discovery in 1985. Fullerene C_{60} is a nanocomposite with 60 carbon atoms, which has been the subject of numerous studies for its ability to store atoms in the interior and accept various structural modifications. The importance of understanding all the possible effects of exposure to fullerenes is very great, since its production and its use is growing every day increasing environmental exposure to this compound. The cholinergic system has as its main neurotransmitter acetylcholine (ACh). The acetylcholinesterase (AChE, EC3.1.1.7) is an important regulatory enzyme that controls the transmission of nerve impulses across cholinergic synapses by hydrolysis of the ACh. AChE levels are controlled by the interaction of ACh with its receptors, and when the interaction is enhanced, the levels of AChE are increased. Therefore, AChE can be used as a marker of cholinergic function. In this study our goal was to determine whether intraperitoneal injections of fullerene C_{60} at the doses of 7.5, 15 and 30 mg/kg and the time of 6h, 12h and 24h of exposure would cause a change in the modulation of the cholinergic system. We observed that the dose of 30 mg/kg in the exposure time of 24 hours showed a 84% increase in enzyme activity compared with the vehicle control group. These results suggest a possible neurotoxic effect. Further studies should be conducted to extend these findings.

Keywords: fullerene C_{60}, zebrafish, Danio rerio, nanotoxicity

LISTA DE ABREVIATURAS

Acetil CoA - acetil coenzima A

ACh - acetilcolina

AChE - acetilcolinesterase

BuChE - butirilcolinesterase

cDNA - ácido desoxirribonucléico complementar

C_{60} – fulereno C_{60}

CHT - transportador de colina

DAG - diacilglicerol

mRNA - Ácido Ribonucléico Mensageiro

PCR - polymerase chain reaction (Reação em Cadeia da Polimerase)

RNA - ácido ribonucléico

SNC - Sistema Nervoso Central

SNP - Sistema Nervoso Periférico

ZFIN - Zebrafish Information Network (Rede Internacional de Dados do Zebrafish)

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CAPÍTULO 1 - INTRODUÇÃO E OBJETIVOS

1. INTRODUÇÃO

1.1 Zebrafish

O *zebrafish* ou peixe-zebra é um pequeno teleósteo (3-4 cm) de água doce, da família Cyprinidae, que vem sendo considerado um modelo ideal para estudos de numerosas doenças humanas (Sloman et al., 2003; Best and Alderton, 2008). George Streisinger foi o pioneiro a estudar esta espécie que, no final da década de 60, aplicou as técnicas de análise mutacional para avaliar o desenvolvimento embrionário do *zebrafish* (Grunwald and Eisen, 2002).

Pode-se observar o interesse pelo *zebrafish* como modelo experimental, através do grande número de laboratórios que o utilizam em suas pesquisas e pelo crescimento exponencial do número de publicações envolvendo esta espécie (Barbazuk et al., 2000; Carvan III et al., 2000; Sprague et al., 2001; Zon & Peterson, 2005; Lieschke & Currie, 2007; Gerlai et al., 2009). São várias as características que favorecem o uso do *zebrafish* como modelo de estudo, tais como: pouco espaço para a sua manutenção, baixo custo, fácil manipulação, desenvolvimento e ciclo biológico rápidos, fácil análise comportamental em um ambiente controlado, boa sensibilidade para drogas, pequeno tamanho e metabolismo rápido (Karlovich et al., 1998; Goldsmith, 2004; Sloman et al., 2003).

O instituto Sanger iniciou o sequenciamento do genoma do *zebrafish* em 2001(Stern & Zon 2003). Seu genoma mitocondrial já é conhecido e vem servindo como base para estudos filogenéticos (Broughton et al., 2001). Os genes desta espécie apresentam grande grau de similaridade com os genes humanos e de camundongos (Barbazuk et al., 2000; Lieschke & Currie, 2007). Nos últimos anos, houve um progresso considerável na genética e genômica

do *zebrafish* (Postlethwait et al., 2000; Amatruda & Patton, 2008; Milan & MacRae, 2008).

O *zebrafish* se tornou o principal modelo experimental para o estudo do desenvolvimento de vertebrados (Anderson & Ingham, 2003). As características básicas de sua embriogênese são bem conhecidas, assim como o destino celular durante o seu desenvolvimento (Kimmel & Warga, 1988; Kimmel, 1989).

Foi criada uma rede de informações na *web* sobre o *zebrafish*, o ZFIN (http://zfin.org), na qual laboratórios do mundo inteiro depositam informações sobre esta espécie (Sprague et al., 2003). Além disso, existe um excelente manual de manutenção e controle das condições de criação deste teleósteo em laboratórios (Westerfield, 2000).

São muitas as áreas para as quais a utilização do *zebrafish* vem se expandindo, tais como biologia do comportamento (Gerlai, 2003; Guo, 2004), toxicologia (Hill et al., 2005), bioquímica (Taylor et al., 2004), neurociências (Edwards & Michel, 2002) e farmacologia (Goldsmith et al., 2004).

Devido as suas peculiaridades reprodutivas e as suas características morfológicas e fisiológicas, esta espécie desperta o interesse pela oportunidade de acelerar o processo da descoberta de novas drogas (Stern & Zon, 2003). Além disto, o *zebrafish* é capaz de absorver, de forma rápida, os compostos que são diretamente adicionados na água e acumulá-los em diferentes tecidos, principalmente no sistema nervoso central (SNC) (Grosell & Wood, 2002). Foram realizados estudos envolvendo aspectos toxicológicos após a exposição a diferentes contaminantes ambientais, tais como a 2,3,7,8-tetraclorodibenzeno-p-dioxina (TCDD) (Dong et al., 2002; Hill et al.,2003), pesticidas carbamatos e organofosforados (Senger et al., 2005), metanol (Rico et al., 2006), etanol (Rico et al., 2008) e metais pesados (Senger et al., 2006a; Rosemberg et al., 2007).

Numerosos estudos avaliando características comportamentais do *zebrafish* estão sendo desenvolvidos (Gerlai et al., 2000; Guo, 2004; Emran et al., 2008; Spence et al., 2008). Alguns estudos observaram a importância do comportamento inato e adquirido em modelos de agressividade, sociabilidade e sua preferência por ambientes claros ou escuros (Serra et al.,1999).

Atualmente, muitos projetos estão sendo realizados com esta espécie com o objetivo de estudar as bases moleculares da neurobiologia, identificando genes envolvidos na formação de circuitos neuronais, no comportamento e nos mecanismos envolvidos na neuropatogênese (Guo, 2004; Eddins et al., 2009; Gerlai et al., 2009). Diferentes sistemas de neurotransmissão já foram identificados nesta espécie tais como: glutamatérgico (Edwards & Michel, 2002; Tabor & Friedrich, 2008), colinérgico (Behra et al., 2002; Clemente et al., 2004; Arenzana et al., 2005; Senger et al., 2006b; Edwards et al., 2007), dopaminérgico (Boehmler et al., 2004; Ryu et al., 2006; Russek-Blum et al., 2008), serotoninérgico (Rink & Guo, 2004; Lillesaar et al., 2007; Norton et al. 2008), histaminérgico (Kaslin & Panula, 2001), gabaérgico (Kim et al., 2004; Delgado & Schmachtenberg, 2008) e purinérgico (Kucenas et al., 2003; Rico et al., 2003; Senger et al., 2004; Low et al., 2008).

1.2 Neurotransmissão Colinérgica

A acetilcolina (ACh) é o neurotransmissor mais importante do sistema colinérgico (Descarries et al., 1997). Sua atividade no SNC é de fundamental importância, pois está relacionada ao comportamento, aprendizado, memória, organização cortical do movimento e controle do fluxo sanguíneo cerebral. (Mesulam et al., 2002; Moretto et al., 2004).

A neurotransmissão colinérgica é fundamental para o correto funcionamento do SNC e representa o sistema neurotransmissor mais antigo filogeneticamente (Gotti & Clementi, 2004). Os neurônios colinérgicos inervam a musculatura voluntária do sistema somático e também são encontrados no SNC (Soreq & Seidman, 2001). A ACh apresenta também uma função neuromoduladora, pois seus níveis regulam a concentração de outros neurotransmissores no cérebro (Cooper et al., 1991). A ACh é formada a partir da Acetil Coenzima A (Acetil CoA), durante o metabolismo celular mitocondrial, e da colina, um importante produto do metabolismo dos lipídeos. A última etapa da síntese ocorre no citoplasma, acumulando o neurotransmissor no interior

das vesículas sinápticas (Kapczinski et al., 2000; Soreq & Seidman, 2001). A colina usada na síntese de ACh pode vir diretamente da reciclagem da ACh, que é hidrolisada pela AChE (acetilcolinesterase) na fenda sináptica ou a partir da fosfatidilcolina. Essas duas fontes de colina são particularmente importantes para o SNC, porque a colina presente no plasma não ultrapassa a barreira hematoencefálica (Taylor & Brown, 1994).

A liberação de ACh depende das variações no potencial elétrico das membranas dos terminais nervosos e este processo é dependente da concentração de cálcio intracelular. Ao ser liberada, a ACh interage com receptores específicos causando despolarização e propagação do potencial de ação na célula pós-sináptica (Oda, 1999). Seus efeitos são mediados pela ativação de receptores nicotínicos e muscarínicos (Soreq & Seidman, 2001; Descarries et al., 1997).

A ACh que permanece na fenda sináptica é hidrolisada por uma colinesterase específica em ácido acético e colina. Grande parte da colina resultante é captada pelo terminal do axônio colinérgico por um transportador de colina (CHT) e reutilizada na síntese de nova ACh (Mesulam et al., 2002) Com base na diferença de afinidade por agentes que mimetizam a ação da ACh, pode-se dividir os receptores em duas classes: nicotínicos e muscarínicos (Tinsley et al., 2004). Os receptores nicotínicos são ionotrópicos e reconhecem a ACh e a nicotina. Estes receptores se localizam no SNC, nos gânglios autonômicos, na junção neuromuscular e na medula adrenal (Sarter & Parikh, 2005). Eles apresentam estrutura pentamérica e estão ligados a canais catiônicos (McKay & Placzek, 2007). Os receptores pertencem a uma família heterogênea que consiste em diferentes subtipos, os quais formam combinações homoméricas ou heteroméricas a partir de 12 diferentes subunidades (α2-α10, β2-β4) (Gotti & Clementi, 2004).

Os receptores muscarínicos são metabotrópicos e ligam-se à acetilcolina e à muscarina. Estão localizados nos gânglios do SNP (Sistema Nervoso Periférico) e nos órgãos efetuadores autonômicos, como as glândulas exócrinas, o cérebro, o coração e o músculo liso (Sarter & Parikh, 2005). Existem cinco subtipos de receptores muscarínicos (M1-M5) que foram clonados e identificados farmacologicamente. Os receptores M1, M3 e M5

estão acoplados a uma proteína Gq/11 e alteram a atividade celular pela estimulação da fosfolipase C, e pela geração do segundo mensageiro IP3, o qual induz a liberação de cálcio intracelular e diacilglicerol (DAG). Contudo, os receptores M2 e M4 estão acoplados a uma proteína Gi que induz sua reposta via inibição da adenilato ciclase (Caulfield & Birdsall, 1998; Uchiyama & Chess-Williams, 2004).

1.2.1 Acetilcolinesterase (AChE, E.C.3.1.1.7)

As colinesterases hidrolisam a ACh na fenda sináptica e desempenham um papel muito importante na neurotransmissão colinérgica, além de outras funções fisiológicas. São classificadas de acordo com suas propriedades catalíticas, especificidade de inibidores e distribuição nos tecidos: A AChE hidrolisa preferencialmente ésteres com grupamento acetil, estando presente principalmente nas sinapses do SNC, SNP parassimpático e junção neuromuscular por outro lado a butirilcolinesterase (E.C.3.1.1.8, BuChe) hidrolisa outros tipos de ésteres como a butirilcolina. Ambas as colinesterases são amplamente distribuídas no organismo (Taylor and Brown, 1999). A AChE é uma importante enzima regulatória que controla a transmissão de impulsos nervosos através da sinapse colinérgica pela hidrólise do neurotransmissor excitatório ACh (Milatovic and Dettbarn, 1996). A AChE é uma serina hidrolase que desempenha um papel essencial no mecanismo colinérgico, catalisando a hidrólise natural do substrato acetilcolina em acetato e colina (Quinn, 1987). Os níveis de AChE parecem ser controlados pela interação da ACh com seus receptores, sendo que quando a interação é acentuada, aumentam os níveis de AChE. No entanto, a AChE pode ser usada como um marcador da função colinérgica, e mudanças na atividade da enzima podem indicar alterações na disponibilidade de ACh e do nível de seus receptores (Fernandes and Hodges-Savola, 1992).

Observa-se a inibição da atividade da AChE quando o *zebrafish* é exposto aos agentes tóxicos paration (Roex et al., 2003) e metanol (Rico et al.,

2006), enquanto que o etanol promove um aumento significativo desta atividade (Rico et al., 2007).

O gene que codifica para a AChE do *zebrafish* foi clonado e sequenciado por Bertrand e colaboradores, em 2001, revelando que esta enzima é codificada por um gene somente e que sua sequência de 634 aminoácidos apresenta 62% de similaridade em relação aos mamíferos. Neste estudo, não foi verificada a presença de um gene que codifique a butirilcolinesterase, indicando que possivelmente não há atividade desta enzima no *zebrafish*.

1.3 Nanotecnologia

A nanotecnologia pesquisa e desenvolve nanopartículas com pelo menos uma dimensão na escala nano, de 1 à 100 nm. Devido a sua composição, tamanho e estrutura, nanomateriais exibem propriedades específicas com aplicações nos campos biomédico, eletrônico e ambiental. Partículas deste tamanho apresentam uma grande área superficial e, frequentemente, exibem propriedades mecânicas, ópticas, magnéticas ou químicas distintas de partículas e superfícies macroscópicas. O aproveitamento dessas propriedades em aplicações tecnológicas forma a base da nanotecnologia de materiais (Quina, 2004).

Nanocompostos derivados de átomos de carbono têm sido foco de interesse para aplicações em vários campos industriais, tais como engenharia eletrônica, produtos farmacêuticos, dispositivos médicos, cosméticos, embalagens de alimentos e outros, desde seu descobrimento em 1985 (Kroto et al. 1985).

Não há dúvida de que a nanotecnologia oferece a perspectiva de grandes avanços que permitam melhorar a qualidade de vida e ajudar a preservar o meio ambiente. Entretanto, como qualquer área da tecnologia que faz uso intensivo de novos materiais e substâncias químicas, ela traz consigo

alguns riscos ao meio ambiente e à saúde humana que ainda precisam ser melhor investigados (Quina, 2004).

1.3.1 Fulerenos

Fulerenos são alótropos moleculares do carbono e existem em várias formas. Foram descobertos em 1985, por três pesquisadores que observavam o grafite sendo aquecido com laser e vaporizando em uma atmosfera de gás hélio. Os átomos de carbono vaporizados se misturaram ao hélio e se combinaram formando agregados moleculares com algumas dezenas de átomos de carbono. Dentre os agregados, duas conformações se apresentaram com alto índice de ocorrência: os compostos com 60 e com 70 átomos de Carbono. Esta avaliação foi feita através de medida com espectrômetro de massa (Kroto et al., 1985 ; Rocha, 1996).

A menor molécula que pode ser formada é a C_{20}, e após ela é a C_{24}. A partir desta última, existem fulerenos em uma sequência com números pares de carbono, ou seja, C_{26}, C_{28}, C_{30}, e assim por diante (Amador, 2006). Até hoje, oito fulerenos estáveis já foram isolados em quantidades significativas, sendo os mesmos denominados [60-*I*h], [70-*D*5h], [76-*D*2], [78-*D*3], [78-*C*2v(I)], [78-*C*2v(II)], [84-*D*2(IV)] e [84-*D*2d(II)] em função do número de carbonos que os formam, do grupo pontual de simetria da molécula e do número do possível regioisômero. Um dentre estes fulerenos, a molécula de simetria *I*h formada por 60 átomos de carbono, o fulereno C_{60} é, sem dúvida, o mais abundante e representativo (Santos et al., 2010).

O que torna o fulereno alvo de inúmeros estudos são suas propriedades como a capacidade de armazenar átomos em seu interior e aceitar diversas modificações estruturais através da adição de radicais e átomos de diferentes elementos, sendo que para cada modificação estrutural suas propriedades se alteram. (Amador, 2006).

1.3.2 Fulereno C_{60}

O fulereno C_{60} (C_{60}) pode ser formado no ambiente, sendo encontrado em fontes antropogênicas, como incêndios florestais e combustão de materiais formados por carbono. Alguns trabalhos mostram o C_{60} presente em amostras de gelo com 10 mil anos de idade (Murr et al., 2004) e em ovos de dinossauro (Wang et al., 1998). Recentemente, foi encontrado em amostras de ar de atmosferas urbanas, demonstrando que os humanos estão expostos ao fulereno no dia a dia (Utsunomiya et al., 2002).

Atualmente, são produzidas toneladas de C_{60} bem como de seus derivados pela adição de grupos funcionais como a hidroxila, carboxila e alquila, entre outros (Borm et al., 2006). Essa grande produção assim como o desenvolvimento do seu comércio, deve resultar na crescente presença dos nanomateriais no ambiente humano, tornando ainda mais importante o entendimento da relação destes nanocompostos com seus locais de destino dentro do organismo, sendo um grande campo para pesquisas toxicológicas (Nakagawa et al., 2011).

O número de átomos de carbono varia de acordo com o tipo de molécula fulerênica estudada, podendo ir de 60 (fulereno C_{60}) a milhares. Estruturalmente, os átomos de carbono se ligam formando hexágonos e pentágonos; o número de pentágonos é de exatamente 12; já, o número de hexágonos varia de acordo com a molécula estudada no C_{60} existem 20 fazes hexagonais (Kroto et al., 1985; Rocha, 1996).

Devido a sua composição e sua conformação estrutural o C_{60} possui uma reatividade alta com os radicais livres chegando a um nível antioxidante muito elevado (Krusic et al., 1991).

Recentemente, derivados do C_{60} demonstraram algumas propriedades biológicas notáveis como promoção de apoptose neural (Dugan et al., 2001), redução da atividade osteoclástica em artrite (Yudoh et al., 2009), atividade protetora contra efeito citotóxico do β peptideo amilóide (Huang et al., 2000; Kim & Lee, 2003; Podolski et al., 2007) e melhora na memória em animais tratados com $A\beta_{25-35}$ (Kim & Lee, 2003; Podolski et al., 2007).

Uma dificuldade particular para estudar o C_{60} em sistemas biológicos é que ele não é solúvel em água (Ruoff et al., 1993). Para superar esta dificuldade, foram desenvolvidos métodos que utilizam solventes orgânicos. Estes métodos criam uma suspensão solúvel em água de C_{60} (Deguchi et al., 2001).

Utilizando estas suspensões solúveis em água, vários estudos mostraram que o C_{60} possui toxicidade para vários organismos. Foi demonstrado que em peixes jovens, a exposição a 0.5 ppm de C_{60} solúvel em água por 48 h, aumenta a peroxidação de lipídios no cérebro e causa depleção de glutationa nas brânquias (Oberdorster, 2004). Além disto, nanocompostos como o C_{60} são lipofílicos, o que lhes permite interagir com membranas de todos os tipos (Foley et al., 2002; Kamat et al., 2000).

Quanto aos mecanismos subjacentes à ação citotóxica, comparando o C_{60} com outro nanocomposto, o fulerol, um ensaio violeta para a viabilidade celular demonstrou que o C_{60} é pelo menos três ordens de grandeza mais tóxico que o fulerol para um fribrossarcoma L929 em camundongo (Isakovic et al., 2006).

2. OBJETIVOS

2.1 Objetivo Geral

Uma vez que (i) o nanocomposto fulereno C_{60}, é usado em nanotecnologia, (ii) apresenta a capacidade de atravessar a barreira hematoencefálica, (iii) a modulação da atividade da AChE tem sido descrita como um marcador de toxicidade em diversas situações e (iv) a neurotransmissão colinérgica é bem conhecida em *zebrafish*, o objetivo deste projeto foi verificar se a administração de fulereno C_{60} poderia alterar a neurotransmissão colinérgica, utilizando-se o *zebrafish* como modelo experimental.

2.2 Objetivos Específicos

Avaliar o efeito da exposição à diferentes doses de fulereno C_{60} sobre a atividade da acetilcolinesterase em homogenato cerebral de *zebrafish* adultos.

Avaliar o efeito de diferentes tempos de exposição ao fulereno C_{60} sobre a atividade da acetilcolinesterase em homogenato cerebral de *zebrafish* adultos.

CAPÍTULO 2 – ARTIGO CIENTÍFICO

Exposure to nano/microparticles of fullerene (C_{60}) increases acetylcholinesterase activity and lipid peroxidation in adult zebrafish (*Danio rerio*) brain

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Exposure to nano/microparticles of fullerene (C_{60}) increases acetylcholinesterase activity and lipid peroxidation in adult zebrafish (*Danio rerio*) brain

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ABSTRACT

Background: Even though technologies involving nano/microparticles have great potential in diverse applications, it is crucial to determine possible toxicity of these technological products before extensive use. Fullerenes C_{60} are nanomaterials with unique physicochemical and biological properties that are important for the development of many technological applications. The aim of this study was to evaluate the consequences of C_{60} exposure in brain acetylcholinesterase expression and activity, antioxidant responses and oxidative damage using adult zebrafish (*Danio rerio*) as an animal model.

Results: None of the doses (i.p.) tested (7.5, 15 and 30 mg/kg) altered AChE activity when zebrafish were exposed to C_{60} during 6 and 12 hours. However, the analysis for 24 hours demonstrated that animals treated with the concentration of 30 mg/kg presented a significant increase in AChE activity (28.54 ± 3.72 µmol SCh.h^{-1}.mg $protein^{-1}$; p = 0.0001) when compared to saline (12.19 ± 0.55 µmol SCh.h^{-1}.mg $protein^{-1}$; p = 0.0001) and to the vehicle control group (15.46 ± 0.57 µmol SCh.h^{-1}.mg $protein^{-1}$; p = 0.0001). The up-regulation of brain AChE activity is not directly related with the transcriptional control. Oxidative damage, measured by lipid peroxidation (TBARS assays) showed a pro-oxidant condition elicited by C_{60} at the highest dose (30 mg/kg) after 24 hours when compared with the vehicle control group (0.11 ± 0.02 vs 0.07 ± 0.01 nmoles/mg of wet tissue; p= 0.0194).

Conclusion: The results presented in this article provided further evidence for neurotoxic effects after C_{60} exposure. In one hand, AChE activity was significantly

enhanced when zebrafish were exposed to C_{60}. Besides, C_{60} showed a pro-oxidant behavior when intraperitoneally injected, indicating toxicity mechanisms other than photo-excitation. Taken together these results suggest neurotoxicity mediated by apoptosis.

Keywords: fullerene C_{60}, zebrafish, acetylcholinesterase, oxidative damage, neurotoxicity

Introduction

Nanoscience investigates nanoscale phenomena and serves as the foundation for nanotechnology, which develops practical applications for nanomaterials (particles typically with sizes in the 1–100 nm range, but not exclusively) [1]. Due to their composition, small size and shape, nanomaterials exhibit novel properties for diverse applications that have already yielded in a variety of commercially available products [2]. As a consequence, it is expected that both humans and environmental systems will be increasingly exposed to nanomaterials in the next years. Nanotoxicology [3] and nanoecotoxicology [4] are emerging disciplines that arose to address the potential human and environmental health effects of nanomaterials exposure.

Fullerenes are molecular materials that call attention after the first preparation of C_{60} which is an allotrope of carbon consisting of 60 carbon atoms joined to form a cage-like structure [5]. C_{60} exhibits unique physical and chemical properties for many technological applications, such as electronics, superconductors, cosmetics [6], and more recently, drug and gene delivery [7]. Although the toxic effects of C_{60} are still mostly unknown, especially those related to neurotoxicity, some general aspects have

already been addressed. For instance, C_{60} are reported to be cytotoxic to various mammalian cells (for review see [8]) and it has been shown to induce lipid peroxidation in human cells [9], in brain of juvenile largemouth bass (*Micropterus salmoides*) and in carp [10, 11]. Moreover, C_{60} was classified as "very toxic" to aquatic organisms by the grid for the potential ecotoxicological hazard evaluation which is based mainly on toxicity to fish, *Daphnia* and algae, and in data about degradability of the substance [4].

In cholinergic neurotransmission, choline acetyltransferase (ChAT) is responsible for the synthesis of acetylcholine (ACh) in the presynaptic neuron. After its release into the synaptic cleft, ACh promotes the activation of metabotropic muscarinic and ionotropic nicotinic cholinergic receptors. The reaction responsible for the maintenance of levels of ACh is catalyzed by two cholinesterases (ChE): acetylcholinesterase (AChE) (E.C. 3.1.1.7) and butirylcholinesterase (BuChE) (E.C. 3.1.1.8) [12]. The zebrafish (*Danio rerio*) has long been considered a powerful animal model for studying several biological events. More recently, zebrafish become also a valuable model to environmental and toxicological studies [13]. It has been demonstrated that BuChE is not encoded in the zebrafish genome, but AChE is encoded by a single gene that has been cloned, sequenced and functionally detected in zebrafish brain [14].

AChE activity has been widely used as a bioindicator of environmental exposure. For example, the inhibition of AChE as a biomarker for assessment of the exposure of organisms to organophosphate and carbamate insecticides is well known (for review see [15]). The inhibition of brain AChE activity of aquatic species by toxic substances such as methanol [16], heavy metals mercury and lead [17] and neurotoxins [18] also has been well established. On the other hand, AChE activation has also been demonstrated

as a consequence of exposure to neurotoxic compounds such as aluminum [19] and ethanol [20].

Therefore, considering that: (i) both humans and environmental systems will be increasingly exposed to C_{60} in a near future, (ii) the neurotoxic effects of C_{60} are far from being completely understood, (iii) measurement of AChE activity in organisms is used worldwide as a biomarker of environmental contamination, (iv) determination of oxidative stress parameters is recognized as a tool to evaluate toxicity-mediated by small particles exposure and that (v) zebrafish is a well-established organism for toxicological analysis, the aim of the present study was to evaluate the effects of C_{60} in brain AChE activity and its gene expression pattern. Furthermore, we have analyzed the effects of C_{60} in parameters related to antioxidant defenses and lipid peroxidation in the brain of zebrafish

Results

Size distribution in suspensions of nano/microparticles

The nano/microparticles mean diameters over the volume showed wide distributions (60 nm – 316 µm; 69 nm – 1,905 µm; 182 nm – 208 µm, for suspensions 7.5, 15, and 30 mg/kg, respectively), with most abundant sizes in the micrometric range (Figure 1A, 1C and 1E). The SPAN values increased with the dilution of suspensions (5.843, 3.003 and 1.607 to suspensions 7.5, 15, and 30 mg/kg, respectively), showing a narrow size distribution with lower dilutions. On the other hand, it is important to emphasize that when considering the mean diameters over the number distribution, we observed that the greater part of particles were under nanometric sizes (Figure 1B, 1D and 1F).

Acetylcholinesterase enzymatic activity and gene expression

The effect of different C_{60} concentrations and times of exposure on brain AChE activity was demonstrated by performing (*in vivo*) experiments using adult zebrafish. None of the concentrations tested (7.5, 15 and 30 mg/kg) altered AChE activity when zebrafish were exposed to C_{60} during 6 hours (Figure 2A) and 12 hours (Figure 2B). However, the analysis for 24 hours demonstrated that animals treated with the concentration of 30 mg/kg presented a significant increase in AChE activity (28.54 ± 3.72 µmol SCh.h^{-1}.mg $protein^{-1}$; p = 0.0001) when compared to saline (12.19 ± 0.55 µmol SCh.h^{-1}.mg $protein^{-1}$; p = 0.0001) and to the vehicle control group (15.46 ± 0.57 µmol SCh.h^{-1}.mg $protein^{-1}$; p = 0.0001) (Figure 2C). The up-regulation of brain AChE activity after exposure to C_{60} (30 mg/kg for 24 hours) could be a consequence of transcriptional control. In order to determine if transcriptional regulation of *ache* has occurred, a qRT-PCR analysis was performed. The results have shown that *ache* transcript levels were not enhanced when compared to the vehicle control group (p = 0.6695; Figure 3) suggesting that the activation of brain AChE is not directly related with the transcriptional control.

In vitro effects of C_{60} suspensions on acetylcholinesterase activity

To verify whether C_{60} nano/microparticles might have a direct effect on the enzyme, we tested the *in vitro* effect of C_{60} suspensions on AChE activity in zebrafish brain. The results showed that C_{60} suspensions did not bring about any alteration in AChE activity (p=0.7701; Figure 4).

Antioxidant analysis

The total antioxidant competence against peroxyl radicals showed an augmented response (lower relative area) in brains of zebrafish exposed to C_{60} (15 mg/kg) for 6

hours when compared to zebrafish exposed to C_{60} (7.5 and 30 mg/kg) for 6 hours (p=0.0209; Figure 5A). No other differences were observed under the experimental conditions. Oxidative damage, measured by lipid peroxidation (TBARS assays) showed a pro-oxidant condition elicited by C_{60} at the highest dose (30 mg/kg) after 24 hours (p=0.0194; Figure 6C).

Discussion

Although technologies evolving nano/microparticles have considerable potential in diverse applications, it is crucial to determine possible toxicity of these technological products before extensive use. Little is known about the toxic effects of fullerenes in brain. At present, only few studies presenting contradictory findings have evaluated possible neurotoxic effects of fullerenes exposure. For instance it was already suggested that C_{60} did not cross the blood-brain barrier [21] whereas the results obtained by Mokrushin [22] suggested that fullerenes possess marked neurotropic properties and are neurotoxic substances irreversibly blocking the electrical activity of the nervous tissue.

Neurotoxicity of C_{60} in fish species has been previously reported. Generation of reactive oxygen species (ROS) by C_{60} is influenced in part by the presence and type of illumination due to the photo-excitation of C_{60} by UV and visible light [11, 23, 24] or even to by-products of the organic solvents employed to prepare C_{60} suspensions [25]. For this reason the C_{60} suspensions were prepared under the protection of light and intraperitoneal injection was adopted as administration route in the *in vivo* protocols, an experimental condition that avoids the influence of light in the analyzed variables. Also, *in vitro* assays were run in darkness.

Suspensions of C_{60} in DMSO were prepared as previously described [26, 27] with modifications. Although DMSO is known to show low toxicity by itself [27, 28], appropriate experimental controls must be employed to eliminate its influence. In this study, the DMSO was diluted to result in 12.5% DMSO as a final concentration. Any signal of toxicity i.e., mortality or even transient alterations in behavior was observed in the vehicle control group (12.5% DMSO). In addition, control group (saline) and vehicle control group were never statistically different in the conditions tested.

The characterization of the size and stability of C_{60} nanoparticles in suspension is very important to evaluate their toxicity once particle size can change during the preparation of the suspension, dilution, and exposure [11]. In this study, the nano/microparticles mean diameters over the volume in the C_{60} suspensions showed wide distributions with most abundant sizes in the micrometric range. The C_{60} has a tendency to form aggregates very easily [7], and this may be a possible cause of this wide distribution. In contrast, the nano/microparticles mean diameters over the number distribution in the C_{60} suspensions demonstrated that the greater part of particles were under nanometric sizes. Totsuka et al. [29] also observed wide distributions by dynamic light scattering in formulations manufactured with C_{60}.

In the present study, we have evaluated the effect of different C_{60} doses (7.5, 15 and 30 mg/kg) and different times of exposure (6, 12 and 24 hours) on AChE activity and *ache* expression in zebrafish brain. In the concentrations tested, only the animals exposed to 30 mg/kg for 24 hours have shown enhanced AChE activity. The qRT-PCR results suggested that the activation of brain AChE is not directly related with the transcriptional control. The *in vitro* results indicated that none of the C_{60} suspensions

had a direct effect on the enzyme. Moreover, we have shown the effects of C_{60} exposure over the antioxidant competence and lipid peroxidation in zebrafish brain. The results also demonstrated that the exposition to 30 mg/kg during 24 hours yielded in higher levels of lipid peroxidation (TBARS). In accordance, Totsuka et al. [29] reported increased micronuclei frequencies, induced DNA damage and increased mutant frequencies after C_{60} nano/microparticles suspension exposure.

AChE is indispensable for terminating acetylcholine-mediated neurotransmission at cholinergic synapses [30]. In this context, AChE is inhibited by organophosphorus and carbamate insecticides and by neurotoxins, which are structural analogues of acetylcholine [31]. In addition, there are evidences to suggest that AChE contributes to diverse physiological processes through its involvement in the regulation of cell proliferation, differentiation and survival. As a consequence, more recently AChE has been redefined as an important regulator of apoptosis, because it can be induced by a variety of apoptotic stimuli ([32]; for review see [33]). It is well known that apoptosis underlies the neurotoxic effects of various compounds. Moreover, zebrafish brain AChE activation has also been demonstrated as a consequence of exposure to known neurotoxic compounds, including aluminum [19] and ethanol [20] and to the cyanobacterial toxin microcystin-LR [34].

The antioxidant or pro-oxidant effects induced by C_{60} exposure is still a debatable issue [9, 24, 25]. C_{60} is photo-excited under UV or visible light [23], a condition for example, that elicited lipid peroxidation in brains of carp [11]. On the other hand, the absence of light did not completely inhibited C_{60} toxicity to embryonic zebrafish [35]. Our results reinforce oxidative stress generation as a pathway of C_{60}-induced toxicity in brain of

fish species, even in the absence of light. Accordingly, it is well known that some apoptotic signals induce pro-apoptotic events increasing ROS in mitochondria. The increased ROS may lead to oxidative stress generation.

Conclusion

The results presented in this article provide further experimental evidence that C_{60} exposure can be neurotoxic. First, brain AChE activity was significantly enhanced when zebrafish were exposed to C_{60}. Besides, C_{60} showed a pro-oxidant behavior when intraperitoneally injected, suggesting toxicity mechanisms that are independent of photo-excitation. Taken together our results suggest neurotoxicity mediated by apoptosis.

Materials and methods

Chemicals

Fullerene (C_{60}, 99.5% purity) was purchased from Aldrich (Milwaukee, WI, USA), DMSO was purchased from Fisher Scientific (Pittsburgh, PA, USA) and Trizma Base, ethylenedioxy–diethylene–dinitrilo–tetraacetic acid (EDTA), ethylene glycol bis(beta amino ethylether)-N,N,N',N'-tetraacetic acid (EGTA), sodium citrate, Coomassie Blue G, bovine serum albumin, acetylthiocholine, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) HEPES, BHT (99%), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (ABAP) and 1,1,3,3-tetramethoxypropane were purchased from Sigma Chemical Co (St. Louis, MO, USA). KCl and SDS (90%) were purchased from Labsynth (Brazil). Tetramethoxypropane (TMP) and 2′,7′-dichlorodihydrofluorescein diacetate were purchased from Acros Organics (Morris Plains, NJ, USA) and Molecular Probes Inc. (Eugene, OR, USA) respectively. $MgCl_2$ and Acetic acid 99.7% were purchased from

Isofar and Vetec (Brazil) respectively. TRIzol® reagent, Platinum® Taq DNA Polymerase and SYBR® Green I were purchased from Invitrogen (Carlsbad, CA, USA). ImProm-II™ Reverse Transcription System was purchased from Promega (Madison, USA). All other reagents used were of analytical grade.

Animals

Adult wild-type zebrafish (*Danio rerio*, Cyprinidae) of both sexes (3-6 months-old) were obtained from a specialized supplier (Redfish Agroloja, RS, Brazil). Animals were kept at a density of up to five animals per liter in 50 L housing tanks with tap water that was previously treated with Tetra's AquaSafe® (to neutralize chlorine, chloramines, and heavy metals present in the water that could be harmful to fish) and continuously aerated (7.20 mg O_2/L) at 26 ± 2 ºC, under a 14/10 h light/dark controlled photoperiod. Animals were acclimated for at least two weeks before the experiments and were fed three times a day with TetraMin Tropical Flake fish food®. The fish were maintained healthy and free of any signs of disease and were used according to the ‘‘Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health. All procedures in the present study were approved by the Animal Ethics Committee of the Pontifical Catholic University of Rio Grande do Sul (PUCRS), protocol number 10/00185-CEUA.

C_{60} suspension

Suspensions of C_{60} in DMSO were prepared as previously described [26, 27] with modifications. Briefly, 7.6 mg of C_{60} was added to 0.5 mL of DMSO and sonicated for 3 h. Following the observation that the particle size was increased over time, the suspension was additionally sonicated for one hour. The suspension of C_{60} was diluted

in water to result in 7.5, 15 and 30 mg/kg suspensions (12.5 % DMSO) that were further sonicated for one hour prior to use. C_{60} suspensions were prepared and stored in a dark condition.

Characterization of C_{60} suspensions

The C_{60} suspensions (7.5, 15 and 30 mg/kg) were characterized in terms of particle size distribution. The mean diameter over the volume and number distribution ($d_{4.3}$) was determined by laser diffractometry (Mastersizer 2000, Malvern Instruments, UK). The value of SPAN was utilized to determine particle size distribution according Eq. (1), where $d_{0.9}$, $d_{0.1}$ and $d_{0.5}$ are the particle diameters determined at 90 %, 10 % and 50 % cumulative undersized volumes, respectively.

(Eq. 1) $$SPAN = (d_{0.9}-d_{0.1})/d_{0.5}$$

Intraperitoneal injection

Intraperitoneal injections were conducted using a 3/10-mL U-100 BD Ultra-Fine™ Short Insulin Syringe 8 mm (5/16″) × 31G Short Needle (Becton Dickinson and Company, New Jersey, USA) according to the protocol established by Phelps et al. [36]. Briefly, the volume injected into the animal was adjusted to the fish bodyweight (mean injection volume was 10 μL) to achieve 7.5, 15 and 30 mg/kg. The animals of the control group received the same volume of saline solution and the animals of the vehicle control received the same volume of 12.5% DMSO. Anesthesia of the animals prior to the injection was obtained by its immersion in a solution of benzocaine (1 mM in MeOH 1%) until the animal showed a lack of motor coordination and reduced respiratory rate. The anesthetized animal was gently placed in a water-soaked gauze-

wrapped hemostat with the abdomen facing up and the head of the fish positioned at the hinge of the hemostat (the pectoral fins were used as a landmark on the abdomen). The needle was inserted parallel to the spine in the midline of the abdomen posterior to the pectoral fins. The injection procedure was conducted in such a way as to guarantee that the animal did not spend more than 10 s out of the water. After the injection, the animals were placed in a separate tank with highly aerated unchlorinated tap water (25 ± 2 °C) to facilitate recovery from the anesthesia. Saline solution was used as control. All the animals that recovered within 2-3 min following the injection continued in the experiment while animals that did not recover during this period were discarded. Six, twelve or twenty-four hours after the injection the animals were euthanized and AChE activity was determined.

In vitro assays of AChE activity

In vitro assays were performed as previously described [37, 38]. Briefly, C_{60} suspension was added to the reaction medium before the pre-incubation with the enzyme-containing lysate from zebrafish brain homogenate and maintained during the enzyme assays. C_{60} was tested at final concentrations of 7.5, 15 and 30 mg/kg. Control treatments with equal volume of vehicle (DMSO 12.5%) were performed to exclude the DMSO effect on the enzyme activities.

Determination of AChE activity

Zebrafish were euthanized and their whole brains were removed by dissection. The brains (two whole brains for each sample) were homogenized on ice in 60 volumes (v/w) of Tris-citrate buffer (50 mM Tris, 2mM EDTA, 2mM EGTA, pH 7.4, adjusted with citric acid), in a glass-Teflon homogenizer. The rate of acetylthiocholine

hydrolysis (ACSCh, 0.88 mM) was assessed in a final volume of 300 µL with 11 mM phosphate buffer, pH 7.5, and 0.22 mM DTNB using a method previously described [39]. Before the addition of substrate, samples containing protein (5 µg) and the reaction medium described above were pre-incubated for 10 min at 25 °C. The hydrolysis of substrate was monitored by the formation of thiolate dianion of DTNB at 412 nm for 2-3 min (intervals of 30 s) in a microplate reader. Controls without the homogenate preparation were performed in order to determine the non-enzymatic hydrolysis of the substrate. The linearity of absorbance against time and protein concentration was previously determined. The AChE activity was expressed as micromoles of thiocholine (SCh) released per hour per milligram of protein. All enzyme assays were evaluated in triplicate and at least four independent experiments were performed.

Gene expression analysis by quantitative real time RT-PCR (qRT-PCR)

Immediately after 24 hours of intraperitoneal injection (described above), the animals were euthanized by decapitation. For each sample, a pool of three zebrafish whole brains was used. Total RNA was isolated with Trizol® reagent (Invitrogen, Carlsbad, California, USA) in accordance with the manufacturer's instructions. The total RNA was quantified by spectrophotometry and the cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega) from 1 µg total RNA, following the manufacturer's instructions. Quantitative PCR was performed using SYBR® Green I (Invitrogen) to detect double-strand cDNA synthesis. Reactions were done in a volume of 25 µL using 12.5 µL of diluted cDNA (1:100 for *EF1α* and *Rlp13α*; and 1:20 for *ache*), containing a final concentration of 0.2 x SYBR® Green I (Invitrogen), 100 µM dNTP, 1 x PCR Buffer, 3 mM $MgCl_2$, 0.25 U Platinum® Taq DNA Polymerase (Invitrogen) and 200 nM of each reverse and forward primers (Table 1). The PCR

cycling conditions were: an initial polymerase activation step for 5 min at 95°C, 40 cycles of 15 s at 95ºC for denaturation, 35 s at 60 °C for annealing and 15 s at 72°C for elongation. At the end of cycling protocol, a melting-curve analysis was included and fluorescence measured from 60 to 99 °C. Relative expression levels were determined with 7500 Fast Real-Time System Sequence Detection Software v.2.0.5 (Applied Biosystems). The efficiency per sample was calculated using LinRegPCR 11.0 Software (http://LinRegPCR.nl) and the stability of the references genes, *EF1α* and *Rlp13α* (*M-value)* and the optimal number of reference genes according to the pairwise variation (*V*) were analyzed by GeNorm 3.5 Software (http://medgen.ugent.be/genorm/). Relative RNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method.

Antioxidant capacity against peroxyl radicals

Total antioxidant competence against peroxyl radicals was evaluated through reactive oxygen species (ROS) determination in tissues samples treated or not with a peroxyl radical generator [40]. Briefly, on a white 96-well microplate, 10 µL of brain homogenates were disposed into the wells, six wells per sample. The reaction buffer (127.5 µL) containing 30 mM HEPES (pH 7.2), 200 mM KCl and 1 mM $MgCl_2$ were added to the wells containing the samples. In three of the six wells of each sample, 7.5 µL of 2,2'-azobis 2 methylpropionamidine dihydrochloride (ABAP; 4 mM) were added. In the other three wells the same volume of ultrapure water was pipetted. After this, the microplate was put into a fluorescence microplate reader (Victor 2, Perkin Elmer), programmed to keep temperature at 35 ºC. At this temperature, peroxyl radicals are produced by thermal decomposition of ABAP [41]. Immediately before microplate reading, it was added in each well 10 µL of the fluorescent probe 2',7'-dichlorofluorescein diacetate (H_2DCF-DA) in a final concentration of 40 µM, according

to the methodology employed by Ferreira-Cravo et al. [42]. H_2DCF-DA is deacetylated and the product H_2DCF is oxidized by ROS to the fluorescent compound DCF, which is detected at wavelengths of 488 and 525 nm, for excitation and emission, respectively. Fluorescence readings (fluorescence units or FU) were performed every 5 min during 30 min. Total fluorescence production was calculated by integrating the fluorescence units (FU) along the time of the measurement, after adjusting FU data to a second order polynomial function. The results were expressed as area difference of FU × min in the same sample with and without ABAP addition and standardized to the ROS area without ABAP (background area). The relative difference between ROS area with and without ABAP was considered a measure of antioxidant capacity, with high area difference meaning low antioxidant capacity, since high fluorescence levels were obtaining after adding ABAP, meaning low competence to neutralize peroxyl radicals [40].

Measurement of lipid peroxidation

Lipid peroxidation was measured through determination of thiobarbituric acid reactive substances (TBARS), following the methodology of Oakes and Van der Kraak [43]. Brain homogenates (10 μL) were added to a reaction mixture made with 150 μL of 20% acetic acid, 150 μL of thiobarbituric acid (2.4%), 50 μL of Milli Q water and 20 μL of sodium dodecyl sulfate (SDS, 8.1%). Samples were heated at 95 °C during 30 min and after cooling by 10 min, 100 μL of Milli Q water and 500 μL of n-butanol was added. After centrifugation (3,000 × *g* during 10 min at 15 °C), the organic phase (150 μL) was placed in a microplate reader and the fluorescence registered after excitation at 520 nm and emission of 580 nm. The concentration of TBARS (nmol/mg of wet tissue) was calculated employing tetramethoxypropane (TMP) as standard.

Protein determination

Protein was measured by the Coomassie blue method [44] using bovine serum albumin as standard.

Statistical analysis

AChE activity and antioxidant analyses were expressed as means ± S.E.M. and analyzed by one-way analysis of variance (ANOVA). Post-hoc comparisons were made using Tukey's test and orthogonal comparisons. Before ANOVA, its assumptions (normality and variances homogeneity) were checked. Molecular data were expressed as means ± S.E.M. and analyzed by Student's *t*-test. In every case the significance level was fixed in 5 % (α= 0.05)

Abbreviations

ChAT: choline acetyltransferase; ACh: acetylcholine; AChE: acetylcholinesterase; BuChE: butyrylcholinesterase; UV: ultraviolet light; ACAP: antioxidant capacity; TBARS: thiobarbituric acid reactive substances; THF: tetrahydrofuran; EDTA: ethylenedioxy–diethylene–dinitrilo–tetraacetic acid; EGTA: ethylene glycol bis(beta amino ethylether)-N,N,N',N'-tetraacetic acid; DTNB: 5,5'-dithiobis-2-nitrobenzoic acid; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BHT: butylated hydroxytoluene; ABAP: 2,2′-azobis(2-methylpropionamidine) dihydrochloride; KCl: potassium chloride; SDS: sodium dodecyl sulfate; TMP: tetramethoxypropane; DMSO: Dimethyl sulfoxide; MeOH: methanol; ACSCh: acetylthiocholine hydrolysis; SCh: thiocholine; ROS: reactive oxygen species.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

GODalF, LWK and RSF performed the AChE experiments. MBA and RSF performed the intraperitoneal injections. TCBP and LWK performed the qRT-PCR experiments. RSB and JMM performed the antioxidant analysis. Analysis of size distribution and agglomeration state of particles was done by ICKG and SSG. CDB, JMM and MRB conceived and supervised the study.

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Figure Legends

Figure 1: Size distribution in suspensions of nano/microparticles. Mean diameters distribution of fullerene C_{60} suspensions (7.5, 15 and 30 mg/kg) were determined over the volume (A, C and E) and over the number (B, D and F) of the nano/microparticles.

Figure 2: *In vivo* AChE activity. *In vivo* AChE activity in zebrafish brain after 06 (A), 12 (B) and 24 hours (C) of fullerene C_{60} exposure at distinct concentrations (7.5 - 30 mg/kg). Bars represent the mean ± S.E.M. of at least three different experiments, each one performed in triplicate. The specific enzyme activity is reported as micromoles of thiocholine released per hour per milligram of protein. The asterisk (*) indicates a significant difference ($p < 0.05$).

Figure 3: qRT-PCR analysis. Relative *ache* expression profile after fullerene C_{60} exposure (30mg/kg for 24 hours) on zebrafish brain. Bars represent the mean ± S.E.M.

Figure 4: *In vitro* AChE activity. *In vitro* effect of different concentrations of fullerene C_{60} (7.5 - 30 mg/Kg) on ACh hydrolysis in zebrafish brain. Bars represent the mean ± S.E.M. of at least three different experiments, each one performed in triplicate.

Figure 5: Antioxidant capacity. Total antioxidant capacity against peroxyl radical in zebrafish brain after 06 (A), 12 (B) and 24 hours (C) of fullerene C60 exposure at distinct concentrations (7.5 - 30 mg/kg). Bars represent the mean ± S.E.M of at least three independent experiments. The asterisk (*) indicates a significant difference when compared 15 mg/kg to 7.5 and 30 mg/kg doses ($p < 0.05$).

Figure 6: Lipid oxidative damage. Concentration of thiobarbituric acid reactive substances (TBARS; nmol/mg of wet weight) in zebrafish brain after 06 (A), 12 (B) and 24 hours (C) of fullerene C_{60} exposure at distinct concentrations (7.5 - 30 mg/kg). Bars represent the mean ± S.E.M of at least three independent experiments. The asterisk (*) indicates a significant difference when compared to the DMSO group ($p < 0.05$).

Table 1: PCR primers design

Enzymes	Primer sequences (5'-3')	GenBank accession number (mRNA)
*EF1α**	F – CTGGAGGCCAGCTCAAACAT	NSDART00000023156
	R – ATCAAGAAGAGTAGTACCGCTAGCATTAC	
*Rpl13α**	F – TCTGGAGGACTGTAAGAGGTATGC	NM_212784
	R – AGACGCACAATCTTGAGAGCAG	
*ache***	F - GCTAATGAGCAAAAGCATGTGGGCTTG	NP_571921
	R - TATCTGTGATGTTAAGCAGACGAGGCAGG	

* According to Tang et al. [45].
** Designed by authors.

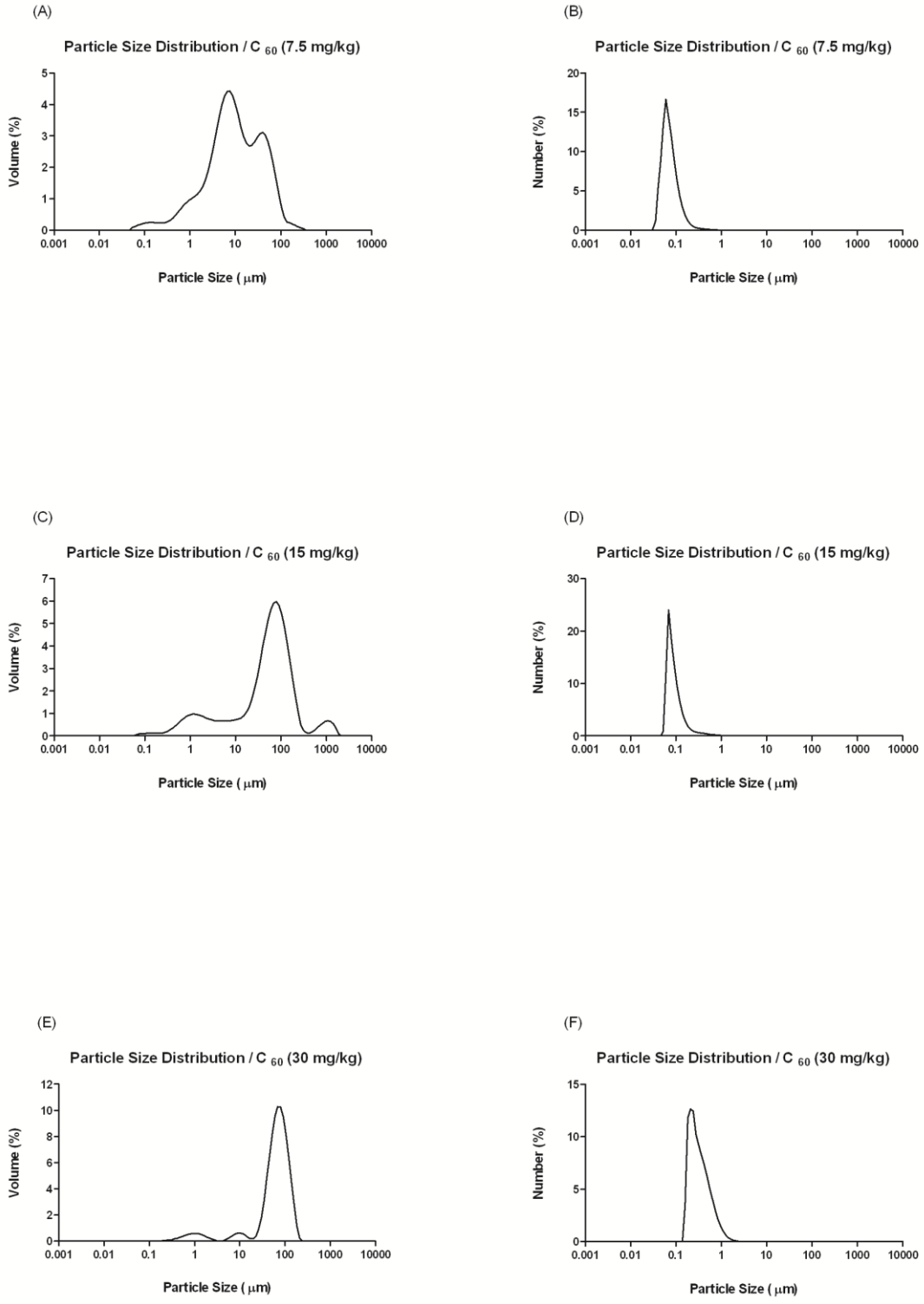

Figure 1 Dal Forno et al., 2011

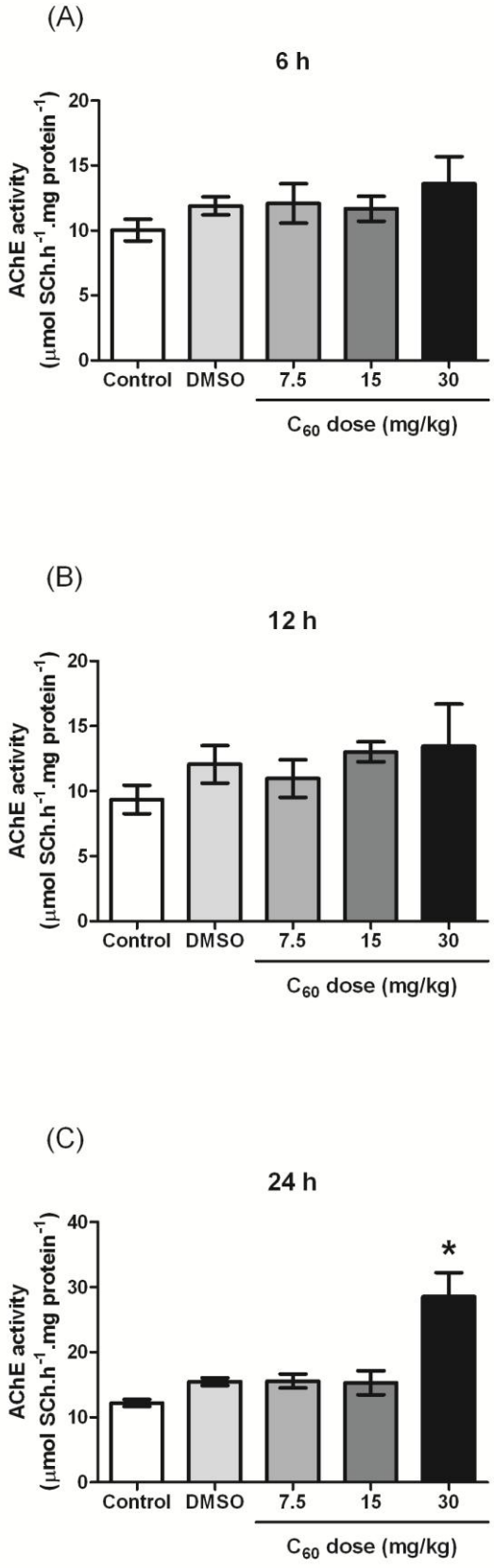

Figure 2 Dal Forno et al., 2011

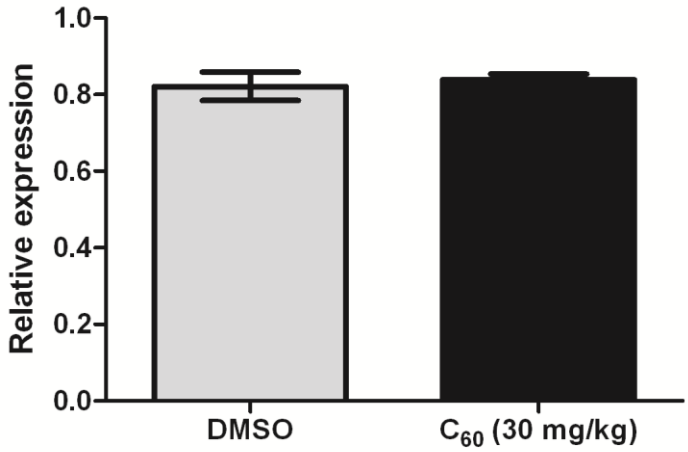

Figure 3 Dal Forno et al., 2011

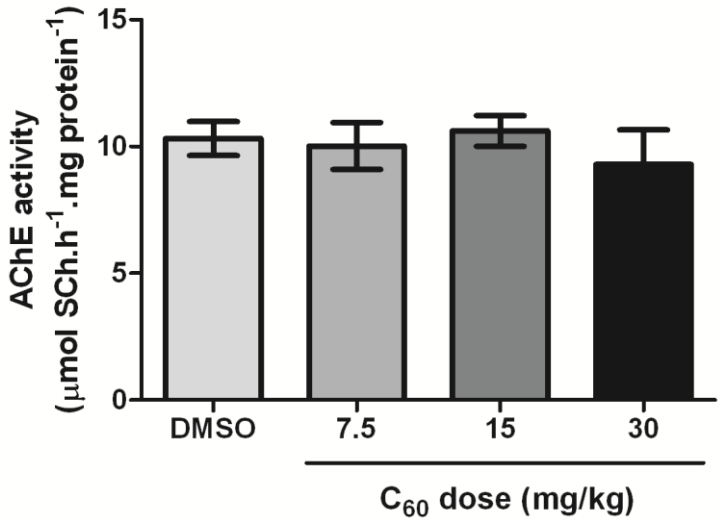

Figure 4 Dal Forno et al., 2011

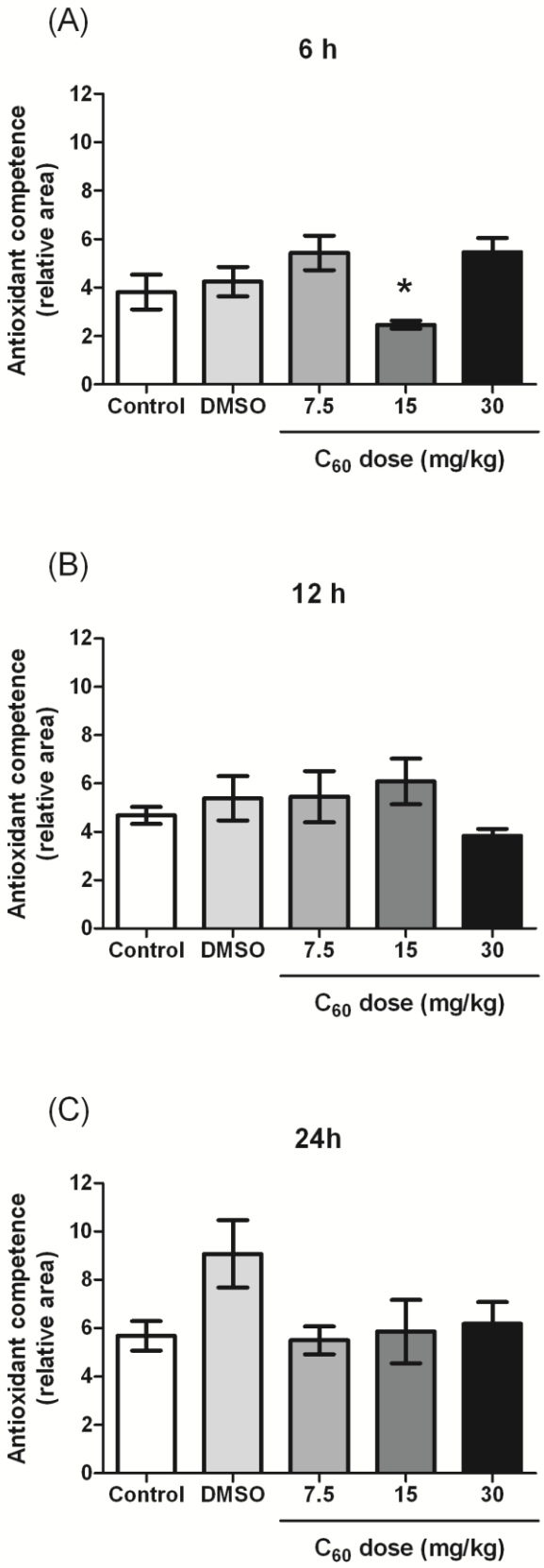

Figure 5 Dal Forno et al., 2011

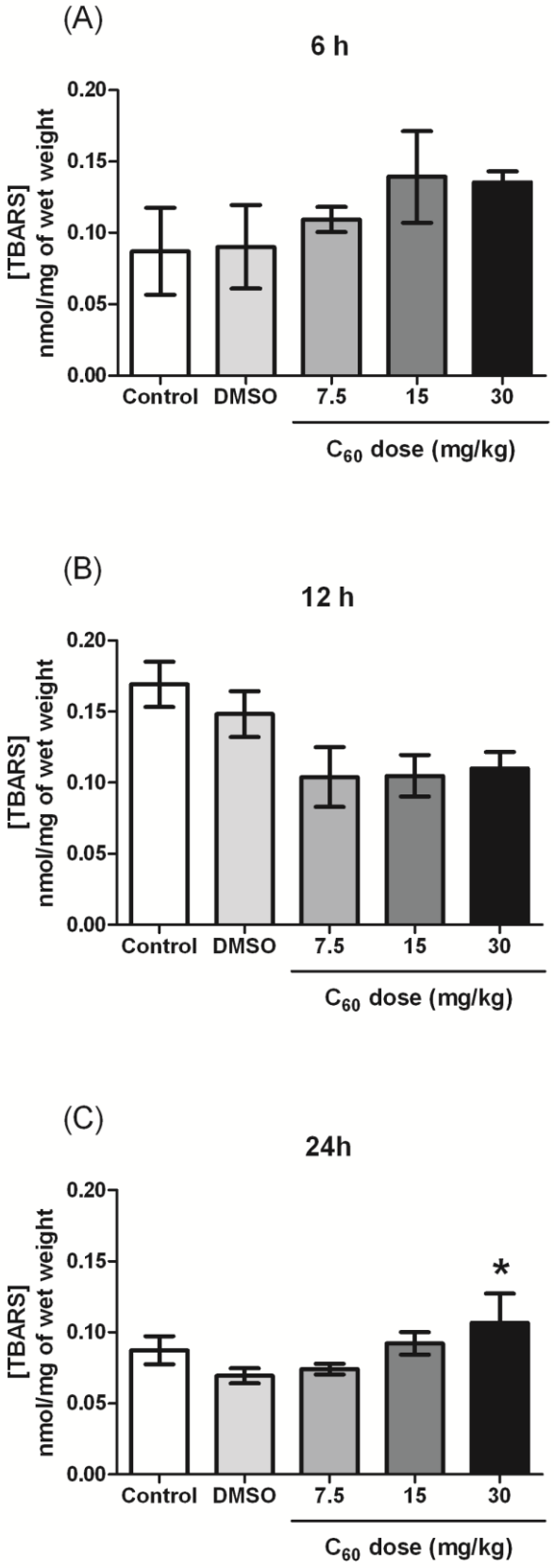

Figure 6 Dal Forno et al., 2011

3. CONSIDERAÇÕES FINAIS

O entendimento sobre efeito de cada nanocomposto nos mais diversos tipos celulares está em pleno desenvolvimento. A importância de entendermos todos os possíveis efeitos de uma exposição aos fulerenos é muito grande, já que a sua produção e sua utilização estão crescendo a cada dia aumentando a exposição do meio ambiente a este composto.

Como vem sendo mostrado em diferentes estudos, a toxicidade do fulereno C_{60} varia de acordo com a linhagem celular estudada. Neste estudo, foi mostrado que quando injetado por via intraperitoneal o efeito do nanocomposto sobre a modulação da neurotransmissão colinérgica, foi dependente da dose injetada, bem como do, tempo de exposição.

Por apresentarem a capacidade de atravessar todo tipo de membrana corporal, os nanocompostos devem ser amplamente testados para ampliar nosso conhecimento sobre as áreas a quais afetam. Devido a sua estrutura, o fulereno C_{60} tem sido alvo para desenvolvimento de novos compostos, adicionando os mais diversos radicais e elementos para observar as alterações resultantes em suas propriedades.

Neste estudo nosso objetivo foi verificar se injeções intraperitoneais de fulereno C_{60} nas doses de 7,5; 15 e 30 mg/kg e nos tempos de 6h, 12h e 24h de exposição causariam alguma alteração na modulação do sistema colinérgico. Observamos que a dose de 30 mg/kg, no tempo de exposição de 24h, apresentou um aumento de 84% na atividade enzimática quando comparado com o grupo controle-veículo. Estes resultados sugerem um possível efeito neurotóxico, embora estudos adicionais devam ser realizados para estender estes achados.

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Anexo I (Comprovante de submissão do artigo científico)

MS: 8074715566403343
Exposure to nano/microparticles of fullerene (C60) increases acetylcholinesterase activity and lipid peroxidation in adult zebrafish (Danio rerio) brain Gonzalo O Dal Forno Jr, Luiza W Kist Dr, Mariana B Azevedo Jr, Rachel S Fritsch Jr, Talita CB Pereira Jr, Roberta S Britto Jr, Sílvia S Guterres Dr, Irene C Külkamp-Guerreiro Dr, Carla D Bonan Dr, José M Monserrat Dr and Maurício R Bogo Dr Particle and Fibre Toxicology

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