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PÓS-GRADUAÇÃO EM CIÊNCIAS FISIOLÓGICAS:
FISIOLOGIA ANIMAL COMPARADA

Influência dos ácidos graxos do tipo ômega-3 ou ômega-6 e o nanomaterial de carbono fulereno (C_{60}) no estado antioxidante em suspensões celulares de cérebro de *Cyprinus carpio* (Pisces, Cyprinidae)

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Dedico este trabalho a minha mãe...

“Eu tenho tanto pra lhe falar

Mas com palavras não sei dizer

Como é grande o meu amor por você

E não há nada pra comparar

Para poder lhe explicar

Como é grande o meu amor por você”

Agradecimentos

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Índice

1 Resumo

Os nanomateriais de carbono como o fulereno (C_{60}) apresenta comportamentos bioquímicos distintos, podendo atuar como antioxidante ou pró-oxidante em diferentes sistemas biológicos. Outra evidência ao C_{60} refere-se a sua característica lipofilica, na qual oferece ação mais direta a diferentes tipos de membranas celulares. Do mesmo modo ácidos graxos poliinsaturados (AGPs) como o ômega-3 (DHA) e o ômega-6 (LA) são importantes para funções celulares da membrana, sendo considerados antioxidantes clássicos. Dessa forma este estudo avaliou em suspensões celulares de cérebro da carpa (*Cyprinus carpio*, Cyprinidae), o efeito de C_{60} após um pré-tratamento com DHA ou LA. Para tal avaliação os ensaios consistiram em um pré-tratamento com AGPs (48h) e após exposição a C_{60} (2h). Como resultados observamos que a viabilidade celular e a capacidade antioxidante total não apresentaram diferença ($p > 0.05$) entre todos os grupos. Em relação a valores de espécies ativas de oxigênio e dano lipídico foi observado redução nos seus valores nos grupos expostos ao C_{60} pré – tratados com AGPs ($p<0.05$). Em termos de cisteína, ocorre uma redução da sua concentração em todos os grupos expostos ao C_{60}. Porém para glutationa a exposição ao C_{60} provoca um aumento de sua concentração nos grupo controle (sem AGPs) e no grupo pré – tratado com DHA. Dessa forma consideramos que o pré – tratamento com AGPs é benéfico às células, uma vez que um aumento nos níveis de glutationa e uma diminuição na concentração de espécies ativas de oxigênio e peroxidação lipídica foram observados nos grupos expostos ao C_{60}. Sendo assim um bom estado nutritivo em termos da concentração de AGPs foi considerado benéfico na exposição ao fulereno.

Palavras chaves: fulereno, ômega-3, ácido docosahexanóico (DHA), ácido linoleico (LA), suspensões celulares, estresse oxidativo.

2 Abstract

Carbon nanomaterials as fullerene (C_{60}) can act as antioxidant or pro-oxidant. Polyunsaturated acids as the omega-3 (DHA) and the omega-6 (LA) are PUFA important for membrane cellular functions. The aim of this study was to evaluate, in cellular suspensions of carp brains (*Cyprinus carpio*, Cyprinidae), the effect of C_{60} after a pre-treatment with DHA or LA. Assays consisted of a pre-treatment with PUFA (48h) and then exposition to C_{60} (2h). Control groups without PUFA or C_{60} were performed. Cell viability and total anti-oxidant capacity did not present difference ($p>0.05$) between all groups. It was observed a reduction ($p<0.05$) of ROS (reactive oxygen species) and MDA (malondialdeyide) concentration in the C_{60} and PUFA groups. Cysteine levels presented a reduction ($p<0.05$) in all groups exposed to C_{60}. For glutathione was registered an increase ($p<0.05$) in C_{60} without PUFAs and in the C_{60} group pre-treated with DHA. Pre-treatment with PUFAs are beneficial to the cells once an increase in the GSH levels and a decrease in the ROS concentration and in a lipidic peroxidation were observed in the C_{60} group. A good nutritive state in terms of high concentration PUFA were shown to be important to confront fullerene exposure.

Key words: fullerene, omega-3, docosaenoic acid (DHA), linoleic acid (LA), cellular suspension, oxidative stress.

3 Introdução geral

O presente trabalho de dissertação foi devenvolvido abordando três perspectivas de estudo. A primeira delas é a geração de espécies ativas de oxigênio (EAO), uma vez que a presença de EAO esta relaciona a importantes processos bioquimícos e fisiológicos. Um segundo assunto abordado, seria o estudo sobre nanomaterias de carbono, em destaque o fulereno (C_{60}), visto o crescimento de insdústrias que utilizam nanomaterias. Cabe ressaltar que a característica lipofilica do fulereno, confere uma atuação mais facilitada em diferentes sistemas biológicos, servindo também como um carreador, por exemplo, de fármacos e xenobióticos. E por fim, iremos apresentar algumas informações referentes a ácidos graxos poliinsaturados (AGPs), uma vez que estes estão presentes em alta concentração em diferentes tecidos e /ou orgãos, em destaque o cérebro.

3.1 Geração de espécies ativas de oxigênio (EAO)

A utilização do oxigênio pelos organismos aeróbicos possibilita uma maior produção de energia proveniente da oxidação dos nutrientes. Porém, durante o processo de redução do oxigênio são geradas naturalmente espécies ativas de oxigênio (EAO) à medida que os elétrons vão sendo repassados ao oxigênio através da cadeia transportadora de elétrons; isto acontece devido a característica do oxigênio de receber um elétron de cada vez (Halliwell e Gutteridge, 1999). As EAO podem ser radicais ou não, sendo que as primeiras possuem um elétron desemparelhado enquanto as espécies não radicalares não possuem esta característica. Na **Tabela 1** são ilustradas as principais espécies ativas considerando radicais e não radicais. Dentro das espécies não radicalares

podemos destacar o peróxido de hidrogênio que é o precursor do radical hidroxila, uma espécie altamente reativa (Halliwell e Gutteridge, 1999).

Diversos estudos mostram que as EAO podem ocasionar danos a distintas macromóleculas como proteínas, lipídeos e DNA (Storey, 1996; Halliwell e Gutteridge, 1999). Além disso, estas espécies podem causar mudanças no sistema de defesa antioxidante como, por exemplo, inibição de enzimas antioxidantes e alterações nos níveis de glutationa (GSH). Quando há um desequilíbrio entre pró e antioxidante em favor dos primeiros caracteriza-se uma situação de estresse oxidativo (Halliwell e Gutteridge, 1999).

Espécies Reativas	
Radicais Livres	**Não Radicais**
Espécies Reativas de Oxigênio (ERO's)	
Superóxido ($O_2^{\bullet}$)	Peróxido de Hidrogênio (H_2O_2)
Hidroxila ($OH^{\bullet}$)	Ácido Hipobromoso (HOBr)
Hidroperoxila ($HO_2^{\bullet}$)	Ácido hipocloroso (HOCl)
Peroxila ($RO_2^{\bullet}$)	Ozônio (O_3)
Alcoxila ($RO^{\bullet}$)	Oxigênio *Singlet* ($^1O_2$)
Carbonato ($CO_3^{\bullet}$)	Peróxidos Orgânicos (ROOH)
Dióxido de Carbono ($CO_2^{\bullet}$)	Peroxinitrito (ONOO)
	Ácido Peroxinitroso (ONOOH)

Tabela 1: Principais espécies reativas incluindo radicais e espécies não radicais (modificada de Barbosa *et.al.* 2008).

Recentemente, Jones (2006) propôs um novo conceito de estresse oxidativo, baseado no fato de um desbalanço de pró-oxidantes e antioxidantes em favor dos oxidantes induzindo a uma alteração na sinalização celular redox.

Para combater os efeitos danosos das EAO, os organismos possuem um sistema complexo de moléculas capaz de interceptar ou degradar estas espécies ou ainda reparar os danos causados por estas. Estas são chamadas de defesas antioxidantes, que podem ser enzimáticas ou não enzimáticas. Dentre as defesas antioxidantes enzimáticas

podemos destacar a atividade de enzimas, como por exemplo, glutationa peroxidase (GPx), glutationa redutase (GR), glutamato cisteína ligase (GCL). A atividade destas enzimas influenciam na concentração de glutationa (GSH) intracelular (Winston e DiGiulio, 1991; Sies, 1993; Griffith,1999).

Dentre as defesas não-enzimáticas podemos então destacar a glutationa reduzida (GSH). A GSH é um tripeptídeo formado por glutamato, cisteína e glicina e representa um dos mais importantes antioxidantes das células. Este tripeptídeo e as enzimas relacionadas à sua síntese, como por exemplo, a GCL formam um sistema atuante no estado redox ideal das células e atua como uma primeira linha de defesa contra as EAO (Shuliga *et al.*, 2002). A **Figura 1** mostra a estrutura química da GSH.

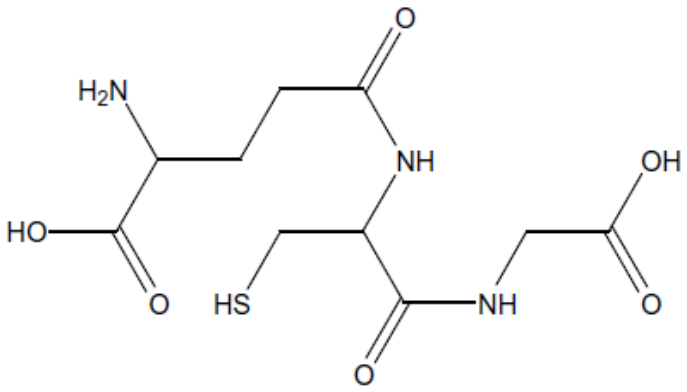

Figura 1 – Estrutura química do tripeptídeo GSH que representa uma das principais linhas de defesa antioxidante das células (retirada de Fraternale *et al.*, 2009).

A manutenção do estado redox de uma célula, como mencionado anteriormente, é fundamental para o funcionamento ideal destas. O estado redox pode ser refletido pela concentração relativa de grupos dissulfetos (S-S-) e grupos tióis reduzidos (-SH) sendo que esta relação é um bom parâmetro para processos bioquímicos, pois está envolvido na estabilização de estruturas de proteínas, funções e transcrição de enzimas, entre outros papéis (Chen *et al.*, 2008).

Os grupos sulfhidrilas e dissulfetos podem estar associadas a moléculas não protéicas (NP-SH) ou a proteínas (P-SH). Dentre os NP-SH podemos destacar a GSH, cisteína (CYS) e a coenzima A (CoA) (Chen *et al.,* 2008), sendo que a GSH é a mais abundante nas células chegando a concentrações milimolares, sendo um parâmetro bioquímica extremamente avaliado para estado redox celular (Winston e DiGiulio, 1991).

3.2 Nanomaterias de carbono: o fulereno (C_{60})

As aplicações de nanomateriais (definidas como partículas de dimensões entre 1-100 nm) nas indústrias têm aumentado consideravelmente nos últimos anos. Suas utilizações são as mais variadas, desde o âmbito farmacêutico até mesmo uso em produtos eletrônicos (Usenko *et al.,* 2008). A expectativa de crescimento da utilização destes nanocompostos pelas indústrias nos próximos anos desperta preocupação, uma vez que não há orientação para possíveis impactos ambientais principalmente em ambientes aquáticos ocasionados pelo processo de nanomateriais manufaturados (Zhu *et al.,* 2008).

O fulereno C_{60} é um nanocomposto formado por 60 átomos de carbono assemelhando-se a strutura de gaiola, amplamente conhecida como “esfera de futebol” (Blickley e Mcclellan-Green, 2008). Na **Figura 2** é ilustrada a estrutura do C_{60}.

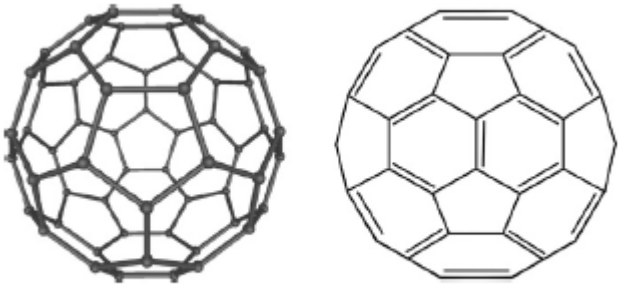

Figura 2: Estrutura molecular do fulereno, composta de 60 átomos de carbono (retirada de Markovic e Trajkovic, 2008).

Do ponto de vista químico o C_{60} apresenta lipoficilidade, o que lhe confere capacidade de interação com diferentes tipos de membranas (Zhu *et al.*, 2006). Assim, sua atuação junto a lipídeos, proteínas e enzimas de membranas celulares pode proporcionar respostas e/ou resultados mais diretos quanto transporte, seletividade e até mesmo fluidez de membranas (Silva, 2005).

Além disso, em termos bioquímicos podemos mencionar dois comportamentos distintos do C_{60}, por vezes agindo como pró – oxidante, por outras como antioxidante (Zhu *et al.,* 2006). No caso de sua ação pró–oxidante ele pode induzir a uma situação oxidativa, devido a sua capacidade de gerar EAO, podendo produzir danos a sistemas biológicos (Usenko *et al*., 2008).

Em um estudo realizado por Zhu *et al.,* (2008) em cérebro de peixe, a exposição ao C_{60} causou dano oxidativo evidenciado pelo aumento da peroxidação lipídica e diminuição os níveis de GSH. Previamente Oberdörster (2004) já tinha evidenciado um aumento nos níveis de peroxidação lipídica em cérebro de peixe exposto ao C_{60}, evidenciando o comportamento pró-oxidante deste nanocomposto neste órgão. Além de danos em lipídeos, o C_{60} pode induzir a inibição da atividade de enzimas de defesa,

incluindo enzimas de reparo do DNA conduzindo ao dano desta macromolécula em diversas espécies (Mashino *et al.,* 2003).

Já em *Escherichia coli,* o efeito pró–oxidante do C_{60} resulta na inibição de metabolismo energético, uma vez que afeta o sistema de transporte biológico de elétrons (Mashino *et al.,*2003). É importante ressaltar que alterações no sistema de transporte de elétrons pode resultar em um déficit energético devido a diminuição na produção de ATP que é fundamental para a manutenção da homeostasia celular.

Por outro lado, existem estudos que relatam um comportamento antioxidante do C_{60} visto que em alguns casos exibe função neuroprotetora agindo na interceptação de EAO (Bosi *et al.,* 2003). Além disso, derivados do C_{60} funcionalizados com grupos carboxil possuem capacidade de neuroproteção caracterizado pela sua eficácia contra morte celular por apoptose, manutenção dos níveis normais de fatores de crescimento, impedimento do bloqueio de receptores NMDA, entre outros (Dugan *et al.,* 2001).

Além de atuar contra EAO em termos de interceptação, há relatos que derivados de C_{60} podem mimetizar a atividade da superóxido dismutase (SOD), uma importante enzima que atua na dismutação do radical ânion superóxidos ($O_2^{\cdot -}$), podendo amenizar possíveis danos por isquemia, desordens neurodegenerativas e doenças inflamatórias que são induzidas principalmente pela produção excessiva deste radical (Ali *et al.,* 2004).

Lee *et al.* (2000) também observaram em mamíferos uma ação benéfica do C_{60} em termos antioxidantes uma vez que este foi capaz de interceptar radicais peroxil evitando a oxidação de lipoproteínas de baixa densidade (LDL). É importante lembrar que o aumento da resistência da oxidação de LDL diminui a incidência de doenças como a aterosclerose.

3.3 Ácidos graxos poliinsaturados – (AGPs)

Os ácidos graxos poliinsaturados (AGPs) são ácidos carboxílicos de cadeia longa com a presença do grupo carboxila (–COOH) e insaturações (duplas ligações). Estas insaturações possibilitam modificações espaciais na cadeia carbonada e interação com diferentes moléculas permitindo que estes AGPs exerçam diferentes papéis (Youdim *et al.,* 2000). A **Figura 3** mostra alguns exemplos de ácidos graxos polinsaturados.

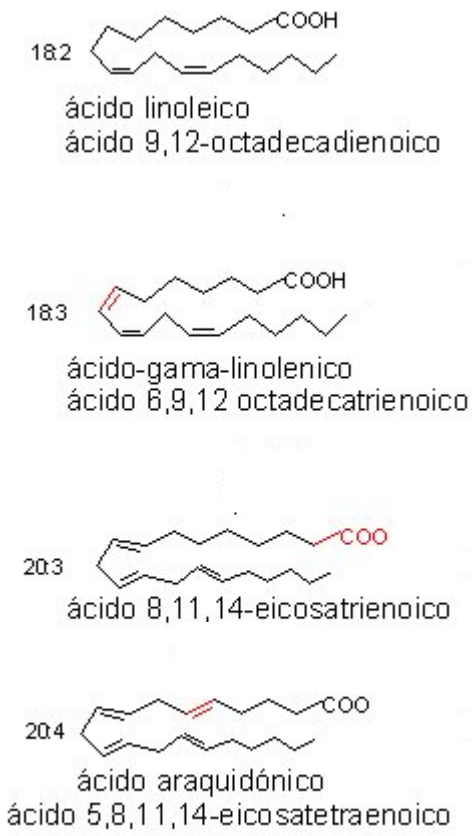

Figura 3: Alguns exemplos de ácidos graxos poliinsaturados AGPs (baseado em Youdim *et al.,* 2000). Os números na parte inferior de cada figura mostram a posição da ligação dupla na cadeia carbonada.

Os ácidos graxos poliinsaturados são importantes em vários processos bioquímicos como balanço energético, biossíntese de membranas e produção de eicosanóides. Nos tecidos, estes AGPs podem ser facilmente oxidados gerando uma quantidade significativa de ATP se comparado, por exemplo, com a oxidação de carboidratos. E esta maior eficiência é devido à molécula de ácido graxo ser altamente reduzida e proporcionar uma maior geração de coenzimas reduzidas e conseqüentemente, mais ATP. Além disso, os ácidos graxos constituem uma forma eficiente de armazenamento de energia sob a forma de trialcilglicerol que pode ser utilizada pelos organismos quando houver aumento na demanda energética através do processo de β-oxidação (Virgili *et al.,* 1996).

Dentre os ácidos graxos insaturados com mais de duas insaturações (poliinsaturados) podem ser destacados os ômega-3 e os ômega-6. As estruturas destes dois AGPs são mostradas na **Figura 4.**

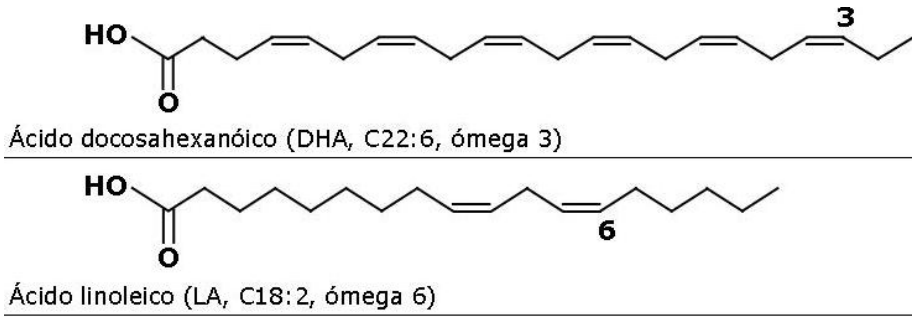

Figura 4: Ácidos graxos poliinsaturados AGPs do tipo ômega -3 e ômega – 6 (baseado em Mahéo *et al.,* 2005). Os números indicados na parte inferior de cada figura representam o número de carbono e o número de insaturações respectivamente.

Os ácidos graxos poliinsaturados são os precursores dos fosfolipídios os quais compõem em sua maioria as membranas biológicas. Sabe-se que quanto maior o grau de insaturação maior a fluidez das membranas sendo esta característica de extrema importância uma vez que a fluidez está relacionada com respostas fisiológicas como

modulação celular, atividade de enzimas e transporte celular entre outros processos (Mahéo *et al.*, 2005). Sendo assim, é provável que o ômega-3 tenha um melhor efeito protetor que o ômega -6 embora o estudo estas características benéficas comparando ambos ácidos graxos ainda sejam escassas em organismos aquáticos.

Podemos ressaltar o cérebro como órgão no qual a presença de AGPs é de fundamental importância, visto que suas membranas apresentam altas concentrações de destes em suas estruturas, sendo considerado um fator limitante nas atividades de receptores de membrana, participando ativamente da sinalização celular (Chen e Subbaiah, 2007).

O ácido docosahexanóico (DHA), por exemplo, é um dos principais neuroprotetores contra dano oxidativo em fotoreceptores, atuando nos processos de transdução de sinais e/ou no desenvolvimento dos cones e bastonetes (Shimazawa *et al.*, 2009).

Korshunov *et al.*, (1998) relatam que a presença de AGPs diminui a geração de EAO em mamíferos. Sabe-se que a presença destes ácidos graxos diminui o índice de doenças cardiovasculares, uma vez que esses ácidos conseguem diminuir os níveis de colesterol no sangue. Além disso, alguns processos induzidos pela produção excessiva de EAO, como a esquemia/reperfusão também podem ser minimizados pela presença de AGPs (McNivena *et al.*, 2004). Tanto o ômega-3 como o ômega-6 podem ser eficientes contra doenças como ateroesclerose visto que diminuem a taxa de oxidação das lipoproteínas de baixa densidade , como já dito anteriormente (Korshunov *et al.*, 1998).

Alguns estudos demonstram que a presença de AGPs podem modular respostas imunes, principalmente os do tipo ômega – 3. Essa ação benéfica se dá principalmente pela estimulação de mediadores antiinflamatórios, nomeadas “resolvins” e “protectins” (Serhan e Savill, 2005).

Além dos efeitos benéficos mencionados anteriormente, a oxidação causada pelas EAO em AGPs pode proteger outras macromoléculas. Virgili *et al.*, (1996) sugere que numa situação oxidativa, o processo de oxidação de AGPs pode ser encarada de maneira positiva, pois assim poderia evitar um dano mais acentuado da célula, como a oxidação de proteínas de membrana evitando assim a perda da funcionalidade destas proteínas que poderiam causar alterações nas repostas celulares.

4 Objetivos

4.1 Objetivo geral

Avaliar o efeito do fulereno (C_{60}) após pré-tratamento a um ácido graxo poliinsaturado (AGPs) do tipo ômega-3 (ácido docosahexanóico - DHA) ou ômega-6 (ácido linoléico - LA) em suspensões celulares de cérebro de carpa (*Cyprinus carpio*, Cyprinidae).

4.2 Objetivos específicos

Determinar, em suspensões celulares de cérebro de *Cyprinus carpio* pré-tratadas com AGPs e expostas ao C_{60} os seguintes parâmetros:

- Viabilidade celular;
- Concentração de espécies ativas de oxigênio (EAO);
- Níveis de glutationa reduzida (GSH);
- Concentração de cisteína (CYS);
- Dano oxidativo em nível lipídico (TBARS);
- Capacidade antioxidante total (ACAP).

5 Justificativas

- O rápido desenvolvimento de nanomateriais nas mais diversas áreas tecnológicas já citadas na **Introdução (Seção 3)**, pode conduzir a uma exposição humana e ambiental destes, cujos efeitos ainda não são completamente conhecidos. Esta questão leva a um problema social-legislativo que gera a necessidade de um processo de avaliação baseado no "Principio de Precaução", pois o conhecimento sobre as necessidades das condições de segurança nos locais de trabalho e a manutenção da saúde ambiental são considerados insuficientes (Rattner, 2004; Barroso Kümmel, 2009). Sendo assim, uma avaliação quanto a toxicidade do C_{60} poderá oferecer dados importantes em termos ecotoxicológicos associado aos segmentos da nanotecnologia. Estudos que avaliem a toxicidade deste nanocomposto poderão contribuir para que se estabeleçam os níveis de concentração que possam ser seguras para o meio ambiente (Usenko *et al.*, 2007).
- Neste estudo foi avaliado o efeito protetor do ômega-3 e ômega-6 à exposição ao fulereno em células de cérebro. Este órgão foi escolhido com base na variedade de informações benéficas existentes sobre o efeito neuroprotetor descritos para estes AGPs (Tapiero *et al.*,2005).
- O $C_{60,}$ como já mencionado é um nanomaterial em destaque entre tantas outros. Contudo muitos estudos relatam seu comportamento danoso, podendo em alguns casos ocasionar injúria celular. Desta forma foi de interesse avaliar a influência dos AGPs nos efeitos induzidos pelo C_{60}. Ainda cabe considerar que a maioria dos estudos descritos na literatura, que envolvem a ação protetora dos AGPs são relatos para mamíferos, pouco se sabe da ação destes em organismos aquáticos, objeto de estudo deste trabalho.

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7 Artigo

Influence of omega-3 or omega-6 fatty acids and the carbon nanomaterial fullerene (C_{60}) on the antioxidant status in fish brain cell suspensions

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Influence of omega-3 or omega-6 fatty acids and the carbon nanomaterial fullerene (C_{60}) on the antioxidant status in fish brain cell suspensions

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Abbreviation: nC_{60}, fullerene; ROS, reactive oxygen species; PUFAs, polyunsaturated fatty acids; DHA, acid docosahexanoic; LA, linoleic; MDA, malondialdeyide; GHS, glutathione; CYS, cysteine.

Abstract

Carbon nanomaterials as fullerene (C_{60}) can act as antioxidant or pro-oxidant. Polyunsaturated acids as the omega-3 (DHA) and the omega-6 (LA) are PUFA important for membrane cellular functions. The aim of this study was to evaluate, in cellular suspensions of carp brains (*Cyprinus carpio*, Cyprinidae), the effect of C_{60} after a pre-treatment with DHA or LA. Assays consisted of a pre-treatment with PUFA (48h) and then exposition to C_{60} (2h). Control groups without PUFA or C_{60} were performed. Cell viability and total anti-oxidant capacity did not present difference ($p>0.05$) between all groups. It was observed a reduction ($p<0.05$) of ROS (reactive oxygen species) and MDA (malondialdeyide) concentration in the C_{60} and PUFA groups. Cysteine levels presented a reduction ($p<0.05$) in all groups exposed to C_{60}. For glutathione was registered an increase ($p<0.05$) in C_{60} without PUFAs and in the C_{60} group pre-treated with DHA. Pre-treatment with PUFAs are beneficial to the cells once an increase in the GSH levels and a decrease in the ROS concentration and in a lipidic peroxidation were observed in the C_{60} group. A good nutritive state in terms of high concentration PUFA were shown to be important to confront fullerene exposure.

Key words: fullerene, omega-3, docosaenoic acid (DHA), linoleic acid (LA), cellular suspension, oxidative stress.

1. Introduction

Nanoparticles have been defined as materials that have a dimension between 1 and 100 nm. These nanomaterials may be present in the environment either in the form of natural sources or in anthropogenic forms (Blickley e Mcclellan-Green, 2008). Due to their small size, these compounds have been used in medicine since they can make it easier the distribution of drugs in the body. Besides that, some nanomaterials have also been widely used in the making of cosmetics and sunscreens (Oberdöster, 2004).

Among nanomaterials, fullerene (C_{60}), composed by 60 carbon atoms that arrange in a stable and symmetrical sphere, has unique chemical and physical features. It lypophilic nature allows it to interact with all types of membranes. Fullerene posses some paradoxical biological effects since in certain situations, acts as a powerful antioxidant, whereas in others, posses pro-oxidant activity (Zhu *et al.*, 2006; Sphon *et al.*, 2009). As a pro-oxidant, it induces the production of reactive oxygen species (ROS), which may affect macromolecules such as lipids, proteins and DNA, characterizing an oxidative stress situation (Barbosa *et al.*, 2008). In addition to that, C_{60} may favor lipidiperoxidation in the presence of light (UV or visible), increasing ROS levels and thus inducing oxidative (Yang *et al.*, 2007; Shinohara *et al.*, 2009).

However, other studies have shown that C_{60} is an expressive antioxidant, employed positively in therapies for some diseases such as Parkinson's disease. Due to the fact that it goes beyond the hemato-encephalic barrier, research on neuro-degenerative diseases have considered relevant the study on fullerene as a neuroprotective agent (Dugan *et al.*, 2001). Furthermore, the high ROS production may also affect the glutamate receptors increasing the inflow of intracellular calcium. In this process C_{60} acts again in a beneficial way, normalizing the calcium concentration, thus assuring the functionality of these receptors (Bosi *et al.*, 2003).

In a study conducted in the liver of rats which were exposed to tetrachloride, the C_{60} also showed to act as an antioxidant. This result was attributed to the C_{60} capacity to intercept ROS (Gharbi *et al.,* 2005).

The paradoxical effect of C_{60} as an anti or pro-oxidant may be associated to several variables such as the solvent used, concentration of C_{60} suspension, as well as to the presence of light. These variables may cause variations in its oxidative beahaviour (anti or pro-oxidant) and, in this way, affecting its toxic properties (Shinohara *et al.*, 2009).

It is known that polyunsaturated fatty acids (PUFA) present a great physiological importance once they act in several key mechanisms for the maintenance, growth and reproduction of many organisms, including fish (Graeff e Tomazelli, 2007). The PUFA may be classified in two big groups, the saturated (without double connections) and the unsaturated (with double connections).

Within the unsaturated (polyunsaturated) of fatty acids of long chain, two families stand out, the omega-3 and the omega-6. Both participate in numerous cellular functions, contributing to the membrane fluidity and also to the activity of membrane enzymes (Mazza *et al.,* 2007). It is known that the docosahexanoic acid (omega 3-DHA) has a positive role within the ocular system, lowering the effects of oxidative stress on photoreceptors, being considered a good neuroprotector. However, studies show that the loss or deficiency in the constitution of PUFAs in the retina may cause failures in the visual acuity (Shimazawa *et al.*,2009).

The cells of the brain membrane (the meninges), for instance, present high PUFA concentrations. Anomalies in the PUFA composition in the cells of these membranes may be inducted by several factors, including ROS production (Shimazawa *et al.,* 2009). Besides, the low concentration of docosahexanoic acid (DHA) and

eicosapentanoic acid (EPA) may induce behavioral disturbances such as depression (Huang *et al.,* 2008)

Besides the above mentioned beneficial effects of the PUFA, some studies suggest that they may act in the protection against oxidative damages (García *et al.*, 2004). However, most studies have described the effects of these PUFA on mammals and little is known about their potential benefits on organs of aquatic organisms exposed to nanoparticles, as in the case of fullerene.

Thus, the aim of this study was to evaluate, in cellular suspensions of carp brains (*Cyprinus carpio*, Cyprinidae), the effect of C_{60} after pre-exposure to a polyunsaturated fatty acid (PUFA) omega-3 type (docosahexanoic acid - DHA) and omega-6 (linoleic acid – LA).

2. Materials and Methods

2.1 Fullerene preparation

Fullerene (C_{60}) suspensions were obtained after shaking 200 mg of pristine C_{60} in 1 L of Milli Q water during two months under constant shaking and under constant fluorescent light at ambient temperature. Afterwards, the suspension was centrifuged at 25,000 x g during 1 h and filtered through a 0.20 µm pore size nylon membrane (Lyon *et al.,* 2006). Fullerene concentration was estimated by measuring total carbon concentration with a TOC-V CPH (Shimadzu) total organic carbon analyzer.

2.2 PUFA preparation

The polyunsaturated fatty acids (PUFA) were obtained from Sigma-Aldrich. The linoleic acid LA (18:2n-6) and the docosahexanoic acid DHA (22:6n-3) were dissolved in absolute ethylic alcohol 0.5% (v/v) and kept in stock solution in PBS at 80ºC. For each experiment, a concentration of 30nM was employed (Serini *et al.,* 2008).

2.3 Cell suspensions

The fish *Cyprinus carpio* L. (Teleostei: Cyprinidae) was used as animal model. The animals were conditioned for a period of at least 14 days, during which they were fed a commercial feed.

In order to prepare the cellular suspensions, each animal was cryo-anesthetized for brain dissection. Afterwards, the brain was washed in PBS- phosphate saline buffer (Ca^{+2} and Mg^{+2} free), prepared with 1% of antibiotics, and subsequentely, a gentle mechanical homogenization followed by a chemical one with tripsyn 0.025% during 15 min. After trypsin treatment, the suspension wa diluted by the addition of, at least, the same volume of Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich). Finally a two-minute centrifugation at 1,100 rpm was done, obtaining the brain cells in the supernatant.

2.4 Exposure of carp brain cellular suspensions to PUFA (omega-3 or omega-6)

In the first stage, called pre-treatment, the cellular suspensions were exposed to the fatty acids. For this, a final concentration of 1.5×10^6 cells /mL was obeyed. Thus, three distinct groups were defined: control group - CT (without PUFA addition); DHA

group (30 nM); LA group (30 nM). All cell groups kere kept at 20 ^{o}C for 48 h without any light.

2.5 Exposure of carp brain cellular suspension to fullerene (C_{60})

In the second stage, called exposure, these pre-treated suspensions were centrifuged for 2 min at 1,100 rpm and immediately the pellets containing the brain cells were ressuspended in Hanks' Balanced Salt Solution (HBSS) and then exposed for 2 hours to the C_{60} (1mg C/L). Thus, and taking into account the pre-treatment and the exposure, the following treatments were defined: CT-CT; CT-C_{60}; DHA-CT; DHA-C_{60}; LA-CT; LA-C_{60}.

2.6 Cell Viability

Cellular viability was assessed by trypan blue (Gibco) exclusion after 50 h (48h of pre-treatment and 2h of exposure). This assay was used to distinguish between proliferation inhibition and cytotoxicity, since the majority of the tests only permit one to observe the number of viable cells. The results obtained in this section indicated the concentration would be used in posterior tests (Trindade *et al.*, 1999).

2.7 Biochemical Analyses

2.7.1 ROS Determination

The determination of reactive oxygen species was performed using with 2',7'-dichlrofluorescein-diacetate (DCF-DA, Molecular Probes) that, in the presence of ROS, generate a fluorochrome measured by fluorescence at wavelength of 488 and 525 nm for excitation and emission, respectively (Ferreira-Cravo *et al.*, 2007). The readings

were carried through in a fluorescence microplate reader (Victor 2 Perkin), in a medium containing 30 mM HEPES (pH 7.2), 200 mM KCl, 1 mM MgCl2, 16 μM DCFDA.

2.7.2 Determination of the total antioxidant capacity

The evaluation of the total antioxidant capacity was determined by the concentration of reactive species of oxygen in the presence and absence of a pro-oxidant to evaluate the competence of the different biological samples in intercepting and/or degrading the added pro-oxidant.

The fluorescence produced in the reading interval (120 min) was calculated after adjusting fluorescence data to a second degree polynomial and integrating the data below the curve. The curves were adjusted to each sample in the absence and presence of peroxi radicals generated by thermo decomposition (35^{o}C) of 2.2´-azobis (2 metilpropionamidina) dihidrocloruro (ABAP; 20 mM; Aldrich) dissolved in phosphate buffer 100 mM (pH 7.4) (Amado *et al.*, 2009).

2.7.3 Thiobarbituric acid reactive substances (TBARS)

Lipid peroxides content was estimated by measuring thiobarbituric acid reactive substances (TBARS), according Oakes and Van der Kraak (2003). Suspension cells were incubated with 20% acetic acid, thiobarbituric acid (0.8%), Milli Q water and sodium dodecyl sulfate (SDS, 8.1 %) at 95 ^{o}C during 30 min and after cooling by 10 min, Milli Q water and n-buthanol was added. After centrifugation (3,000 x g during 10 min at 15 ^{o}C), the organic phase (150 μl) was placed in a microplate reader and the fluorescence registered after excitation at 515 nm and emission of 553 nm. The concentration of TBARS was calculated employing a standard curve of tetramethoxypropane (TMP, Acros Organics).

2.7.4 Measurement of reduced glutathione and cysteine

Some of important redox state parameters (Jones 2006a, b) considered were: reduced glutathione (GSH) and cysteine (Cys) concentrations. Levels of GSH and Cys were simultaneously measured through an HPLC system (Rodríguez-Ariza *et al.*, 1994) consisted of one ESA584 pump (ESA, Bedford, USA) connected to a quaternary solvent delivery system (ESA 582LPG) and a Prominence DGU-20A5 degasser system (Shimadzu, Kyoto, Japan). This system was coupled to a Coulochem III (ESA, Bedford, USA) electrochemical detector (EC) for the detection of the compounds with a guard cell (M5020) set at +900 mV and a double analytical cell (M5011) maintained at +650 mV (first cell, for GSH and Cys). The thiolic compounds were separated through an ACE5-C18 column (25 cm x 4.6 mm , 5 um), before their detection on the EC detector. The mobile phase was consisted of sodium phosphate 50 mM , pH 2.7 and 50 uM octanesulfonic acid with 2% acetonitrile, and was pumped isocratically at 1 mL/L. The signal of the peaks was recorded and monitored on an EZChrom Elite software. Quantification of the compounds in samples was done based on standard calibration curves previously constructed for each compound.

2.8 Statistical Analyses

The data obtained in the different dosages were tested as to their normality and homoscedasticity, through Shapiro-Wilks and Levene tests, respectively (Zar, 1984). Afterwards, they were compared through the ANOVA test for independent samples, by comparing the average of the sampled groups using a significance level of 5% (α= 0.05).

3. Results

In terms of viability it observed not significant changes after treatment with LA or DHA and C_{60} ($p>0.05$) (**Figure 1**).

In the control group co-exposure to C_{60} it was observed a significant lowering of reactive oxygen species (ROS) concentration when compared with control group not exposed to C_{60} ($p<0.05$). In the groups pre-treated with LA or DHA no significant alteration was registered after C_{60} exposure ($p>0.05$) (**Figure 2**).

The GSH levels in the CT-C_{60} group showed a significant increase when compared to CT-CT group ($p<0.05$). The same result was observed in the group pre-treated with DHA. However, in the group pre-treated with LA no significant increase was observed after exposure to C_{60} ($p>0.05$) (**Figure 3a**).

In terms of cysteine levels, the CT-C_{60} group presented a significant decrease when compared with CT-CT group ($p<0.05$). These same results were observed in the both groups pre-treated with LA or DHA (**Figure 3b**).

The total antioxidant capacity was not altered significantly in any group after C_{60} exposure neither after pre-treatment with both PUFA ($p>0.05$) (**Figure 4a**).

Finally, the TBARS content was not modified after exposure to nC_{60} in the both control group ($p>0.05$). This same result was observed in the group treated with DHA. While in the group treated with LA there was a significant decrease in the group co-exposed to nC_{60} ($p<0.05$) (**Figure 4b**).

4. Discussion

Some studies have suggested that fullerene may, in some situations; act as a pro-oxidant and, in others, as an anti-oxidant. By the other side, it is known that PUFA are important as neuro-protectors (Spohn *et al.,* 2009; Shimazawa *et al.,* 2009).

As alterations in terms of cellular viability were not observed by the Trypan blue method, it is concluded that fullerene does not induce the cellular death at used concentrations.

In the present study, it was observed significant reduction of ROS concentration in the cells exposed only to the C_{60} in relation to the control group, both without PUFA pre-treatment ($p<0.05$). The PUFA groups without exposure to C_{60} showed similar comportment, DHA and LA groups there was a reduction in the ROS concentration ($p<0.05$). A similar result was observed in a study conducted by Shimazawa *et al.* (2009) in which the DHA reduced the ROS levels in ganglionic cells of the retina. When the cells were pre-treated with both PUFA and then exposed to C_{60} did not show significant difference in relation to their respective PUFA control groups. By the other side, the groups that received the pre-treatment with both PUFA and was exposed to C_{60} are compared to the ones that received just the C_{60}, it may be observed that there was a reduction in the ROS concentration ($p<0.05$). This result suggests the importance of the pre-treatment with PUFA, making the brain cells become less vulnerable to ROS attacks, potentiating the membrane structuring, fluidity and/or permeability, being able to avoid posterior alterations of its functionality.

The total anti-oxidant capacity of the system did not present significant difference after pre-treatment with PUFA, nor after exposure to C_{60}. In mammal's cells, Andrievsky et al., (2009) observed that C_{60} induced an increase in the total anti-oxidant capacity after the 15 min of irradiation with a lethal dose of X-rays (7 Gy).

Nonetheless, the MDA concentration (malondialdehyde), a product of the lipidic peroxidation process, indicated a significant drop in the concentration of this compound when comparing the control group ($p<0.05$) to the ones that have the pre-treatment of PUFA. A similar result was also observed by Gutierrez *et.al.*, (2007), in which the presence of PUFA induced a decrease in the lipid peroxidation of brain and liver of birds. The group pre-treated with omega-6 and exposed to the C_{60}, however, generated a higher drop in the MDA concentration, increasing the anti-oxidant behavior of this PUFA when comparing to the respective control group. Contrary to our results, in a study conducted by Sayes et al., (2005), evaluating the C_{60} effects on different cellular types (human dermal fibroblasts and neuronal human astrocytes), a significant increase in the lipid peroxidation was observed, meaning a pro-oxidant effect of fullerene (at a concentration of 100 mg/L).

In terms of the GSH levels, it was observed a significant increase of this anti- in C_{60} without the PUFA pre-treatment and in the C_{60} group pre-treated with DHA. Parallel to this result, as mentioned previously, the levels of lipidic peroxidation were lower in the group C_{60} when pre-treated with DHA, indicating the protective effect of GSH against lipid peroxidation. The high value of MDA in group pre-treated with LA and exposed to C_{60} fitted with the lower GSH content in this group. These results suggest that the increase of the GSH levels improved the detoxificating capacity of the cells, diminishing the lipidic damage mainly in the group treated with DHA.

The maintenance of the cellular redox state is characterized by the rate of reduced (SH) and oxidated (S-S) tiols groups and this maintenance is responsible for the normal functioning of many biochemical processes, including enzyme activity and protein structure, among others (Chen and Subbaiah et al., 2007). In this study, a significant reduction in the cysteine concentration in all the groups that were exposed to the C_{60} was observed, regardless of the pre-treatment. This results points that C_{60} interfered in the cellular redox state, mainly in group LA.

. However, for GSH, the C_{60} exposure induced an increase of this trypeptide in the C_{60} group and in the C_{60} pre-treated with DHA. This result may suggest that the fullerene induced an unfavorable situation to the cell, once a decrease in the cysteine concentration was observed. Yet, this condition may have been minimized by the observed increase in the GSH levels in this work. In fact, no oxidative damage in terms of MDA was observed after the exposition to the fullerene, suggesting that cysteine is being employed for GSH synthesis.

Based on the results obtained in the present study, it may be suggested that pre-treatment with PUFA are benefic for carp brain cells once an increase in the GSH levels and a decrease in the ROS concentration and in a lipidic peroxidation were observed. The exposure to C_{60} showed to aid the antioxidant responses triggered by PUFA, as showed by in group control (without treated) exposed to fullerene and in group pre-treated with DHA exposed to fullerene, that promoted augmented intracellular GSH levels when compared with group control (without treated and exposed). Also, C_{60} exposure lowered MDA levels, and this effect was re-inforced by LA pre-treatment. Future works are necessary to consolidate these results, where it was registered that omega-3 and omega-6 were shown to be important in the modulation of the antioxidant responses. Fullerene appears to play an antioxidant role if considered its effects on

MDA levels. Its putative effect on GSH synthesis, lowering intracellular cysteine concentration needs confirmation in order to give more one evidence of the antioxidant response triggered by this carbon nanomaterial.

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Figure legends

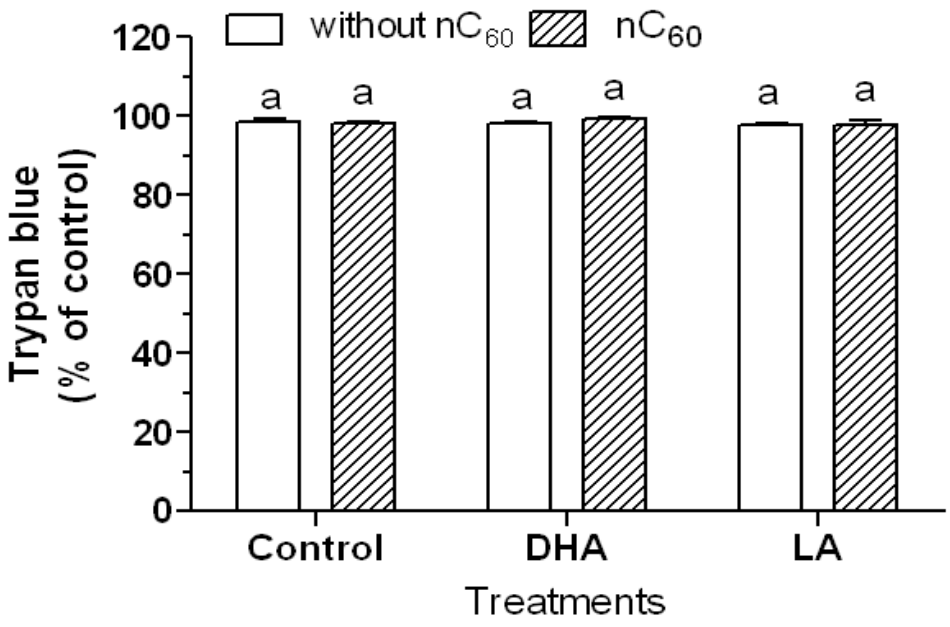
Figure 1. Cell viability in cellular suspention of *Cyprinus carpio* L. (Teleostei: Cyprinidae) pre-treated with fatty acid (LA or DHA) and without fatty acid and them all exposed to $C_{60.}$ Data are expressed means ±1 SE (n=3). Differents letters indicate significant differences ($p<0.05$) with respect to control treatment. Time of exposition was 50h (48h of pre-treatment and 2h of exposure).

Figure 2. Reactive oxygen species (ROS) concentration (area) in cellular suspention of *Cyprinus carpio* L. (Teleostei: Cyprinidae) pre-treated with fatty acid (LA or DHA) and without fatty acid and them all exposed to $C_{60.}$ Data are expressed means ±1 SE (n=3). Different letters indicate significant differences ($p<0.05$) with respect to control treatment. Time of exposition was 50h (48h of pre-treatment and 2h of exposure).

Figure 3. (a) Reduced glutathione (GSH) levels (ng GSH/ 20µl of cellular suspension). **(b)** Cysteine levels (Cys) (ng Cys/ 20 µl of cellular suspension) in cellular suspention of *Cyprinus carpio* L. (Teleostei: Cyprinidae) pre-treated with fatty acid (LA or DHA) and without fatty acid and them all exposed to C_{60}. Data are expressed means ±1 SE (n=3). Different letters indicate significant differences ($p<0.05$) with respect to control treatment. Time of exposition was 50h (48h of pre-treatment and 2h of exposure).

Figure 4. (a) Total antioxidant capacity against peroxyl radical (relative area). **(b)** TBARS content (nmol of MDA/ 100 µl of cellular suspension) in cellular suspention of *Cyprinus carpio* L. (Teleostei: Cyprinidae) pre-treated with fatty acid (LA or DHA) and without fatty acid and them all exposed to C_{60}. Data are expressed means ±1 SE (n=3). Different letters indicate significant differences ($p<0.05$) with respect to control treatment. Time of exposition was 50h (48h of pre-treatment and 2h of exposure).

Figure 1.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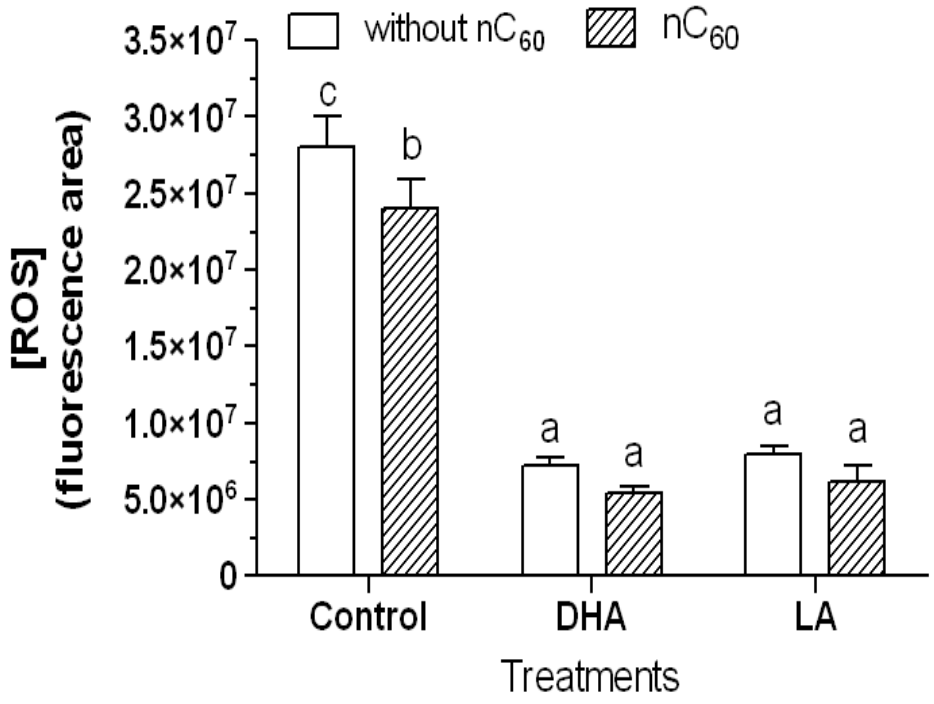
Treatments

Figure 2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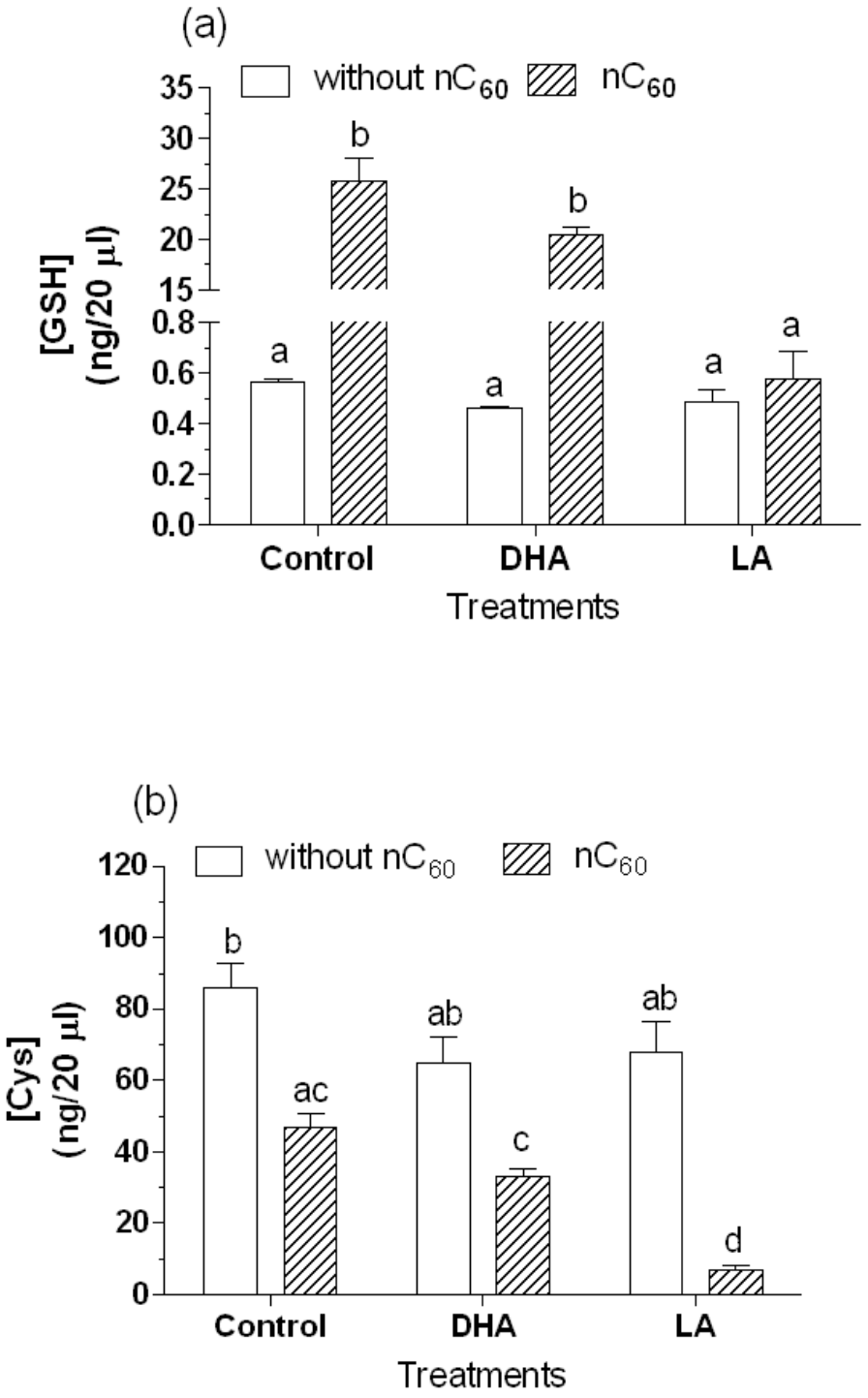
Treatments

Figure 3.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DHA
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Treatments
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without nC60
nC60
[Cys] (ng/20 µl)
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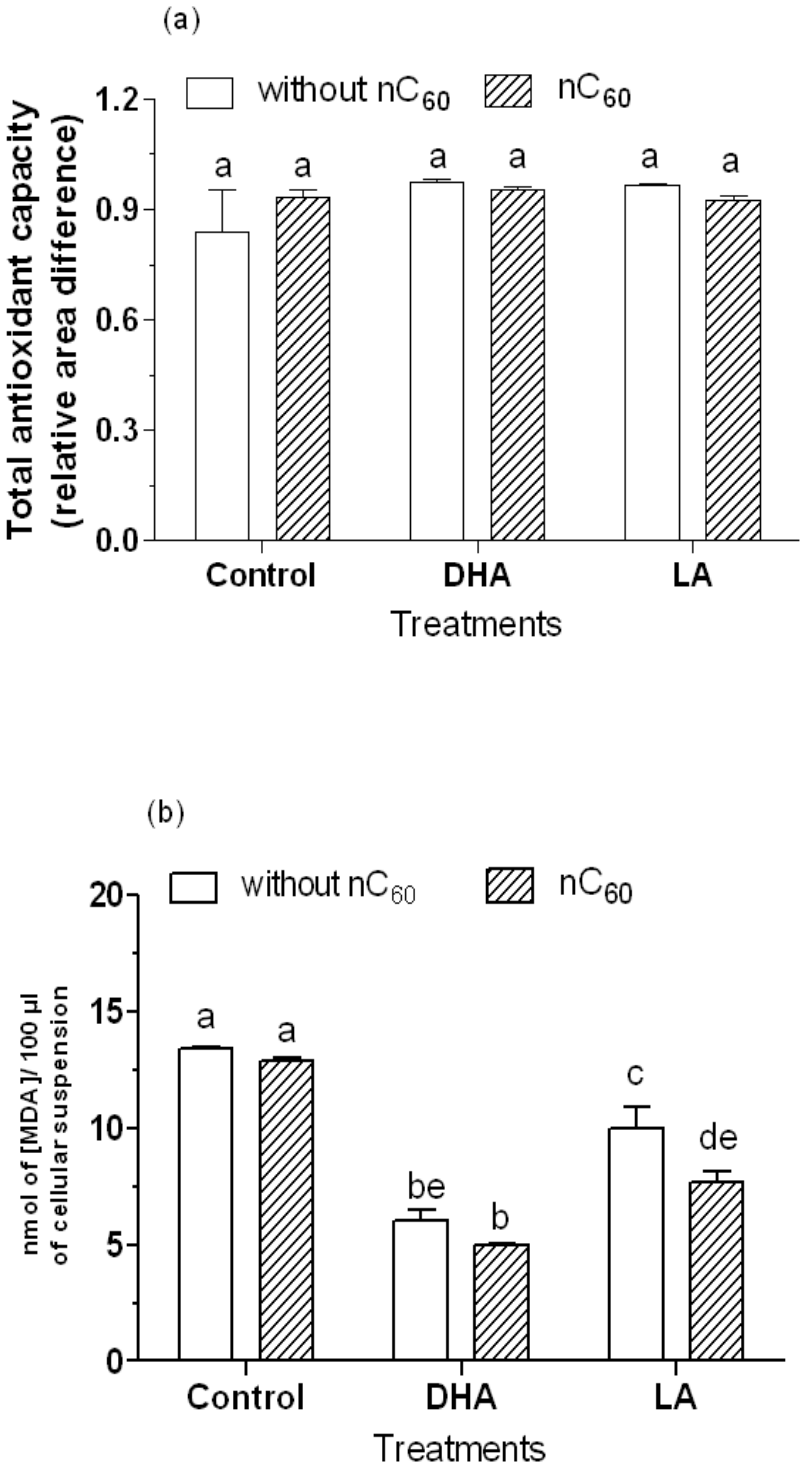
Figure 4.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Treatments
(b)
without nC60
nC60
nmol of [MDA]/ 100 µl of cellular suspension
20
15
10
5
0
a
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Control
DHA
LA
Treatments

8 Conclusões gerais

- Em termos de viabilidade celular não houve mudanças após o pré – tratamento aos AGPs, nem a exposição ao $C_{60;}$
- O grupo $C_{60,}$ sem pré-tratamento aos AGPs, apresentou uma diminuição significativa na produção de EAO a respeito do grupo controle sem pré-tratamento aos AGPs e sem exposição ao C_{60}. Já os grupos pré-tratados com AGPs não apresentaram diferenças quanto expostos ao C_{60} em termos da produção de EAO. Os grupos pré-tratados com AGPs e expostos ao C_{60} mostraram uma diminuição significativa na produção de EAO respeito do grupo exposto ao C_{60} sem pré-tratamento com AGPs.
- Os níveis de GSH foram aumentados perante a exposição ao C_{60} no grupo sem pré-tratamento aos AGPs e no grupo pré – tratado com ômega-3 (DHA). Este aumento pode ter contribuído para diminuição de EAO observado nos mesmos grupos;
- Para os níveis de cisteína a exposição ao C_{60} em todos os grupos ocasionou uma queda. Esta queda pode estar associada a uma alteração no estado redox das células ou, alternativamente, estar envolvida na síntese de GSH;
- Em termos de danos oxidativos nos lipídios a exposição ao C_{60} com pré-tratamento aos AGPs provocou uma queda de concentração de MDA quando comparado ao grupo exposto ao C_{60} sem pré-tratamento aos AGPs. Da mesma forma houve uma diminuição significativa nos grupos pré-tratados com os AGPs e sem exposição ao C_{60} respeito do grupo controle (sem pré-tratamento e sem exposição ao C_{60}). Pode se observar que houve uma menor concentração de MDA no grupo pré-tratado com DHA. Estes

resultados sugerem que o pré-tratamento com AGPs e principalmente com DHA minimiza o dano oxidativo nos lipídeos;

- Com os resultados mostrados na presente dissertação, podemos concluir que o C_{60} oferece alguma mudança no estado redox intracelular quando observamos as concentrações de GSH e CYS. Porém não podemos afirmar que essas mudanças foram danosas, uma vez que não se observou dano lipídico ou alterações na capacidade antioxidante total. De fato a combinação de LA e C_{60} demonstrou diminuir ainda mais os níveis de MDA, sugerindo um papel antioxidante do fulereno;
- Baseado nestes resultados podemos inferir que o pré-tratamento com ambos AGPs foram eficientes para reforçar a proteção das células de cérebro do peixe *Cyprinus carpio* em termos de dano oxidativo, uma vez que o fulereno por si só já apresentou efeitos antioxidantes.

9 Perspectivas futuras

O presente trabalho mostrou que o pré-tratamento com ácidos graxos de tipo ômega-3 e ômega-6 fornecem proteção em nível de dano oxidativo em termos de lipoperoxidação e que este efeito é modulado pelo fulereno no caso da pré-exposição com LA.

Trabalhos futuros poderiam continuar a explorar as repostas antioxidantes associados a um estado nutricional rico em ômega-3 e/ou ômega-6, analisando com maior detalhe o efeito modulador do fulereno nestas respostas. Ou ainda usar uma concetração de C_{60} que seja pró – ocidante e ver o quanto LA e DHA são capazes de reverter essa ação danoso do fulereno.

10 Anexo I: Normas da Revista Food and Chemical Toxicology

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General Information: Food and Chemical Toxicology publishes papers that fulfil the views as laid down in the Aims and Scope section. The Journal's main purpose is the publication of papers reporting and interpreting original unpublished toxicological research, particularly studies promoting an understanding of the mechanisms underlying toxic effects or improvements in methods for predicting adverse effects. Papers reporting the toxicological examination of specific foods, chemicals or consumer products will be published, irrespective of the positive or negative nature of the results, provided the tests and reporting meet current standards of acceptability. In addition, Brief Communications (see description below) will also be considered, as will concise interpretative Reviews of toxicological topics of contemporary significance. Letters to the Editor will be limited to comments on contributions already published n the journal; if a letter is accepted, a response (for simultaneous publication) will be invited from the authors of the original contribution.

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