Universidade Federal do Rio Grande – FURG
Programa de pós-graduação em Ciências Fisiológicas
Fisiologia Animal Comparada
Laboratório de Neurociências

Análises Bioquímicas e Comportamentais do Fulereno C60 em cérebro de ratos: efeitos relacionados ao tamanho de partícula

Ândrea Kraemer Barbosa

Dissertação defendida no âmbito do programa de Pós-Graduação em Ciências Fisiológicas:
Fisiologia Animal Comparada
Como parte dos requisitos para obtenção do título de MESTRE em
Fisiologia Animal Comparada

Orientador: Profª. Drª. Daniela Martí Barros

Rio Grande – RS

2010

“Feliz aquele cujo conhecimento é livre de ilusões e superstições.”

Buda

Agradecimentos

Em primeiro lugar a minha família, sendo o meu pai João Cândido o alicerce para que hoje eu esteja onde estou. Também é importante salientar que sempre foi meu porto seguro, onde sempre pude recorrer nos momentos difíceis.

Ao meu amigo e noivo Fabrício, que foi meu companheiro nas horas boas e ruins, sempre me ajudando nos momentos em que mais precisei.

A minha mãe, que sempre está de alto astral dando um ânimo quando eu me sentia para baixo.

A querida Cleuza, que além de sogra sempre foi uma grande amiga, me ajudando e aconselhando, sendo uma personagem fundamental no meu amadurecimento.

A professora Daniela, que foi uma verdadeira orientadora, tanto no lado científico quanto no emocional, sendo de suma importância para meu crescimento.

Ao meu amigo e colega Gustavo Parfitt, que foi de extremo companheirismo para que este trabalho fosse concluído com êxito.

A minha amiga e "co-orientadora" Juliane, que com a sua experiência me ajudou muito na confecção desta dissertação.

A todos meus colegas e amigos do laboratório de neurociências, Dadá, Renan, Lidiane, André e Fernanda, que foram muito importantes, deixando o ambiente de trabalho um lugar feliz e saudável de se trabalhar.

A todos que não citei, mas que sabem que ocupam um lugar muito importante na minha vida.

Ao curso de Pós-Graduação em Ciência Fisiológicas, onde pude desenvolver meu trabalho, adquirir novos conhecimentos e fazer amigos .

Ao CNPQ e a CAPES pelo auxílio financeiro e pela bolsa.

Sumário

Lista de Abreviaturas

Corpo da dissertação

BDNF – Fator Neurotrófico Derivado do Cérebro
C_{60} – Fulereno
CA-1 – Corno de Amon
EAO – Espécie Ativas de Oxigênio
GCL – Glutamato Cisteína Ligase
GSH – Glutationa
LAM – Labirinto Aquático de Morris
nC60 – nanocomposto Fulereno
NGF – Fator de Crescimento Neuronal
NT-3 – Neurotrofina 3
NT-4 – Neurotrofina 4
SNC – Sistema Nervoso Central
TBARS – Substâncias Reativas ao Ácido tiobarbitúrico
UV – Radiação Ultra-violeta

Artigo

ABAP – 2,2′-azobis 2 methylpropionamidine dihydrochloride
AD – Alzheimer Disease
AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
BDNF – Brain -derived Neurotrophin Factor
CA-1 - *Cornu Ammonis*
CCl4 - Carbon tetrachloride
CTR – Control group
DPBS – Dulbecco's Phosphate Buffered Saline
ELISA – Enzyme-linked immunosorbent assay
EPSP – Excitatory postsynaptic potential
GCL – Glutamate Cysteine Ligase
GSH – Glutathione
H2DCF-DA – 2',7'- dichlrofluorescein-diacetate
HDF – Human dermal fibroblast
HepG2 – Human liver carcinoma cells
HRP – Horseradish Peroxidase
LPO – Lipid peroxidation
LTP – Long Term Potential
MDA – Malondialdehyde
nC60- nanoC60 – Fullerene
NDA – Naphthalene dicarboxialdehyde
NHA – Neuronal human astrocytes
NMDA - *N*-Methyl-D-aspartic acid
pAb – Policlonal Antibody
PUFAs – Polyunsaturated Fatty Acids
ROS – Reactive Oxygen Species
SOD – Superoxide dismutase
TBARS – Thiobarbituric acid reactive substances
TMB – Tetramethoxypropano

TOC-V CPH – Total Organic Content
TQ – Target Quadrant
UV – Ultraviolet light
WM – Water Maze
γ-GC – γ - glutamylcysteine

Resumo

A nanotecnologia é uma área em franca expansão, mas apesar da crescente produção mundial de nanocompostos pouco se sabe sobre os mecanismos de interação com os sistemas biológicos. Atualmente, o fulereno C_{60}, é um nanomaterial muito utilizado na indústria, sendo, produzido em escala industrial. Neste trabalho analisamos os efeitos que esse composto, em diferentes tamanhos de partícula, pode exercer em cérebro de ratos Wistar machos, em relação a parâmetros comportamentais, níveis de BDNF e estresse oxidativo no hipocampo. Os animais tratados com o Fulereno no maior tamanho de partícula (0.45µm) demonstraram prejuízo em relação ao aprendizado e, conseqüentemente, na memória espacial na tarefa comportamental Labirinto Aquático de Morris (LAM), o que pode estar relacionado à diminuição significativa dos níveis da neurotrofina BDNF no hipocampo destes animais. Este mesmo tamanho de partícula pareceu ter uma ação antioxidante *per se*, uma vez que, a concentração de espécies ativas de oxigênio (EAO) foi diminuída, capacidade antioxidante total foi aumentada e não houve dano lipídico, porém, um parâmetro de sistema antioxidante endógeno (GCL e GSH) não foi alterado. Portanto, a partícula de maior tamanho parece ter agido como uma esponja de radicais. O menor tamanho de partícula (0.20µm) analisado pareceu não ter prejudicado o aprendizado e a memória espacial no LAM, sendo os níveis de BDNF sem diferença significativa em relação ao grupo controle; também não apresentou ação antioxidante tão evidente como o Fulereno 0,45µm, salvo, a capacidade antioxidante total, que teve uma diferença significativa, assemelhando-se com o tratamento de maior tamanho. Portanto, como nosso experimento, pode-se observar que o Fulereno é um composto muito peculiar, uma vez que, a mesma substância em diferentes tamanhos de partícula possui efeitos distintos em cérebro de ratos expostos a esse nanocomposto.

Palavras- Chaves: Nanotoxicologia, Fulereno, memória espacial, BDNF, hipocampo e estresse oxidativo.

Abstract

Nanotechnology is a rapidly expanding area but despite the increasing world production of nanocompounds, the interaction mechanisms with the biological systems are poorly known. Nowadays, the fullerene production has been increasing considerably, being industrially fabricated. In our research we use males Wistar rats exposed to two different fullerene suspensions, with 0,2µm and 0,45µm particle size, to analyze in relation behavioral parameters, BDNF levels and oxidative stress in rat hippocampus. The bigger particle size impaired learning and consequently spatial memory on the Water Maze (WM) task, and showed significant decrease on BDNF levels in the hippocampus. This particles size demonstrated an antioxidant action, because, the Reactive Oxygen Species (ROS) has decrease, the antioxidant capacity was increase and no showed lipid peroxidation action. Perhaps, this antioxidant action can be related to Fullerene sponge capacity, because, this treatment do not change GCL activity and GSH concentration. Animals that received treatment with the 0,20µm particle size not showed impairment on learning or spatial memory, the BDNF levels are similar to control group. This treatment not demonstrated an antioxidant action, just in antioxidant capacity analyzes, and was this 0,20µm particles showed results similar to treatment with the bigger particle. Therefore, the Fullerene are a peculiar nanomaterial, because, in the same analyzes this showed a different actions relation a particles sizes in the same environmental experiment.

Key Words: Fulerene, spatial memory, BDNF, hippocampus and oxidative stress.

Introdução

Desde a antiguidade já se tem idéia da presença das nanopartículas. Demócrito de Abdera (470-370 aC) já dizia que a matéria era constituída por elementos indivisíveis e invisíveis a olho nu, os então chamado átomos.

Atualmente, já se tem um grande conhecimento em relação à existência de partículas imensamente pequenas, sendo possível a manipulação e até mesmo a criação dessas.

Pode-se definir nanociência como sendo a área do conhecimento que estuda os princípios fundamentais das moléculas e estruturas, nas quais pelo menos uma das dimensões está compreendida entre cerca de 1 e 100 nanômetros, sendo esse a bilionésima parte de um metro. Em 1974, Norio Taniguchi cunhou o termo "nanotecnologia", que abarcava em seu significado máquinas que tivessem níveis de tolerância inferiores a um mícron (1000 nm) (Alves, 2004) e atualmente é a ciência baseada no estudo e na construção de nanopartículas.

Nos dias de hoje, países como Estados Unidos, Europa e Japão têm incentivado pesquisas nesta área a fim de promover e ampliar a utilização de nanopartículas em aplicações comerciais (Thomas et. al., 2006), tais como, filtros, opacificadores, catalisadores, semicondutores, microeletrônicos, cosméticos e carreadores de drogas (Nel et al, 2006).

A nanotecnologia se utiliza da capacidade que as substâncias em escala de nanômetros têm de interagir com o ambiente que a cerca, onde há um aumento significativo da relação superfície/volume e características físicas e químicas muito peculiares (Oberdörster et al., 2005), bastante distintas daquelas verificadas em materiais que se apresentam em partículas em escalas maiores .

Crescentes preocupações sobre o impacto desses materiais sobre a saúde humana e do meio ambiente iniciaram os primeiros estudos em profundidade a respeito do efeito da exposição de nanomateriais aos sistemas biológicos, gerando uma nova área do conhecimento, a nanotoxicologia, (Oberdörster, 2005). Embora, seja uma área de suma importância, o desequilíbrio entre o conhecimento que se tem dos nanomateriais e seus eventuais efeitos tóxicos pode ser claramente visualizado baseando-se em

uma análise realizada na base de dados SCOPUS (www.scopus.com), utilizando as palavras chaves "nanotechnology" e "nanotoxicology".

Tabela 1. Documentos publicados e indexados na base scopus sob as palavras chave nanotechnology e nanotoxicology em 28 junho de 2008 e em 30 de junho de 2010.

	Nanotechnology	Nanotoxicology
28 de junho de 2008	33.578	111
30 de junho de 2010	52.352	182

As pesquisas na área de nanotecnologia estão em franca expansão, no entanto, os estudos com relação à nanotoxicologia não acompanham as pesquisas até o momento realizadas.

Assim detectando a escassez de estudo nanotoxicológicos, este experimento pretendeu contribuir para o esclarecimento e geração de conhecimentos no que diz respeito aos possíveis danos causados pelo nanomaterial Fulereno, especialmente, relacionados à saúde.

Histórico do Fulereno

Cálculos teóricos realizados desde 1966 já mostravam que a existência de gaiolas estáveis formadas exclusivamente por átomos de carbono era possível (Hirsch, 2005). Em 1985, experimentalmente, Kroto e colaboradores comprovaram existências destas gaiolas. Nesse experimento, uma placa de grafite foi submetida a um laser pulsado de alta freqüência o que gerou agregados que foram analisados por espectrometria de massas. Neste experimento houve a formação de moléculas grandes constituídas exclusivamente por átomos de carbono, com fórmula Cn onde n= 30 - 190, sendo C_{60} e C_{70} os mais abundantes. Em condições específicas para a formação destes agregados, o espectro de massas apresentou o pico do C_{60} como o mais expressivo, apresentando-se como uma molécula excepcionalmente estável e simétrica, sendo chamada pelos pesquisadores de *fullerene* em homenagem ao arquiteto americano Buckminster Fuller, idealizador dos domos geodésicos, cuja forma arquitetônica segue o mesmo princípio de simetria e estabilidade. Sir H. W. Kroto, R. F. Curl e R. E. Smalley, com este experimento, procuraram

mimetizar condições interestelares para comprovar a existência de grandes e excepcionais cadeias de carbono no espaço. Em 1996 essa nobre descoberta foi agraciada com o Nobel de Química.

A molécula do nC_{60}, mesmo rica em elétrons, pode se comportar como uma espécie eletronegativa capaz de captar, de modo reversível, até seis elétrons e conseqüentemente formando os ânions correspondentes (Echegoyen e Echegoyen, 1998) que foram comprovados por cálculos teóricos.

Em meio aquoso, estas moléculas formam colóides, alguns na escala de nanômetros. Como em qualquer composto, essas associações coloidais conferem-lhe uma grande relação superfície/volume potencializando a sua reatividade com biomoléculas (Oberdörster et al., 2005). Além disso, sua característica apolar permite a passagem pelas membranas biológicas, razão pela qual tem representado uma promissora ferramenta na biomedicina, como por exemplo, transporte de medicamentos, de efeitos radioterápicos e das utilizadas para diagnósticos por imagem; atividade anti-viral, através da inibição do acesso a enzimas virais ao substrato pelo preenchimento da cavidade hidrofóbica dos sítios catalíticos; terapia fotodinâmica através da produção de oxigênio singleto e de outros radicais livres; atividade anti-microbiana por intercalação e desestruturação de membranas celulares (Bosi, 2003).

Memória, Estresse Oxidativo e Fulereno

Podemos afirmar que somos aquilo que recordamos, literalmente. Não podemos fazer aquilo que não sabemos como fazer, nem comunicar nada que desconheçamos, isto é, nada que não esteja na nossa memória. O acervo de nossas memórias faz com que cada um de nós seja o que é. Um ser para o qual não existe outro igual. Portanto, podemos afirmar que o quão grave é um composto cuja ação seja deletéria à memória.

A memória é a habilidade que os organismos têm de guardar, reter e evocar informações, sendo um processo fisiológico necessário para a sobrevivência e interação desses com o ambiente (Izquierdo and Medina 1997; Kandel 2001; McGaugh 2000). Sendo, então, um bom parâmetro para se avaliar condição cerebral (Dorman, 2000).

Tanto para a memória de curta duração quanto para a de longa duração uma gama de respostas é necessária. Há inúmeras moléculas envolvidas nas ditas sinalizações intracelulares que acontecem frente a estímulos, como por exemplo, segundos-mensageiros que aumentam muito as respostas que um neurônio pode apresentar a um estímulo sináptico, seja com a ativação de quinases que podem amplificar e prolongar sinais mediante a fosforilação de outras proteínas, bem como a promoção da síntese de proteínas, através da modulação da transcrição genômica, que é uma resposta dependente da atividade, o que leva a alterações duradouras na função celular chamada de plasticidade neuronal (Kandel 2001). Para que se tenha sucesso no aprendizado e na memória é de extrema importância que se tenha alterações a curto e longo prazo em sinapses individuas entre neurônios.

As *neurotrofinas* são proteínas essenciais para o desenvolvimento, função, sobrevivência e plasticidade neuronal (Sofroniew et al., 2001; Huang e Reichardt, 2001; Lu et al., 2005; Lipsky e Marini, 2007) . No Sistema Nervoso Central (SNC) dos mamíferos as principais neurotrofinas são: neurotrofina -3 (NT-3), neurotrofina-4 (NT-4), fator de crescimento do nervo (NGF) e fator neurotrófico derivado do cérebro (BDNF), sendo esta última a de maior interesse para estudos relacionados ao aprendizado e memória (Alonso et al., 2002, 2005). O BDNF no hipocampo está envolvido não somente na consolidação da memória (Alonso, et al., 2002) como também na reorganização neuronal dependente da atividade que pode estar diretamente envolvida na formação da memória (Alonso et al., 2004). Existem crescentes evidências indicando que alterações no BDNF e aumento do estresse oxidativo podem estar envolvidos na fisiopatologia de transtornos mentais (Kapczinski et al., 2008).

Na literatura científica há resultados conflitantes em relação à verdadeira ação do fulereno frente a sistema biológicos. Kamat e colaboladores (1998) foram os primeiros a demonstrar outra característica do fulereno, pois até então, esse nanomaterial era sempre citado como um potente antioxidante. Ele observou que o fulereno sob a forma de seu complexo ciclodextrina tinha ação oxidativa em microssomas de ratos, quando exposto a radiações não ionizantes, neste caso, luz visível e ultravioleta. Mostrou que houve danos em termos de peroxidação lipídica através do ensaio com substâncias reativas ao ácido tiobarbitúrico (TBARS), gerou hidroperóxidos lipídicos, dienos conjugados e danos protéicos, que foram avaliados pela

carbonilação de proteínas e perda das enzimas ligadas à membrana celular; todos estes eventos compõem o chamado cenário de estresse oxidativo.

Sayes et al, (2005) demonstrou com seus experimentos que o fulereno age como uma substância citotóxica em linhagens celulares de fibroblastos, hepatócitos e astrócitos sendo a sua toxicidade causada por peroxidação lipídica.

O cérebro é um dos mais suscetíveis a sofrer dano oxidativo devido a sua alta atividade metabólica, que induz um alto consumo de oxigênio (35 mL/min/kg), e pela presença em grande quantidade de ácidos graxos poli-insaturados nas membranas celulares e que podem sofrer oxidação (Packer et al., 1997; Cardozo-Pelaez et al., 2000; Qiao et al., 2004). Além disto, existe abundância de metais de transição com atividade redox como o ferro e níveis relativamente baixos de defesas antioxidante em algumas regiões do cérebro como hipocampo e córtex, o qual contribui à vulnerabilidade que este órgão apresenta frente à ação de pró-oxidantes (Balu et al., 2005).

Xiao (2005) encontrou que o fulereno atua como uma substância de atividade neuroprotetora, pois é capaz de reagir com espécies ativas de oxigênio (EAO) através de sua capacidade de esponja de radicais. Este composto faria a limpeza de EAO, diminuindo quantidade desses radicais em níveis celulares, sendo, portanto, um potente antioxidante e conseqüentemente, protegendo as células humanas e de outros mamíferos de danos causados por estresse oxidativo.

A ação do Fulereno em cérebro de mamíferos tanto na presença quanto na ausência de luz visível e UV até agora é desconhecida. Sendo então, o estudo de sua ação em tecido nervoso central de suma importância.

Objetivos

Objetivos gerais

Este projeto objetivou avaliar os efeitos do nanocomposto fulereno (C_{60}) sobre os processos de aprendizado e memória espacial correlacionando-os com análises de estresse oxidativo e níveis de BDNF.

Objetivos Específicos

Avaliações comportamentais:

- Determinar o efeito do fulereno (C_{60}) sobre o aprendizado e memória espacial na tarefa comportamental Labirinto Aquático de Morris (Water Maze)

Avaliações Bioquímicas:

- Analisar a capacidade do fulereno em induzir estresse oxidativo no hipocampo de ratos;

- Avaliar a potencialidade do fulereno (C_{60}) em alterar os níveis da neurotrofina BDNF em hipocampo de ratos.

Artigo

Revista: Biomaterials
(Fator de impacto: 7,7)

Fullerene (C_{60}) actions upon spatial memory, oxidative stress and BDNF levels: effects related to particles size

Ândrea, Kraemer[1,2], Gustavo Parfitt[1,2], Daiane Acosta [1,2], Juliane Ventura-Lima[1,2],

José María Monserrat [1,2], Daniela Martí Barros[1,2]*

[1]Instituto de Ciências Biológicas (ICB), Universidade Federal do Rio Grande - FURG, Rio Grande, RS, (Código postal) Brazil

[2]Programa de Pós-Graduação em Ciências Fisiológicas - Fisiologia Animal Comparada (FURG).

Corresponding author: Daniela Martí Barros

Phone/Fax: +55 53 32935170

E-mail: barrosdm@yahoo.com.br

Abstract

Nanotechnology is a rapidly expanding area but despite the increasing world production of nanocompounds, the interaction mechanisms with the biological systems are poorly known. Fullerene C_{60} (nC_{60}) is the most widespread and well-studied member of the new class of carbon nanoparticles with unique physicochemical and biological properties. In our research we use two different fullerene suspensions, with 0,2µm and 0,45µm particle size. Wistar rats were anesthetized and bilaterally implanted cannulae above the hippocampus CA1 area. After (48h) recovery, they were treined in a water maze task during five days, after the test day, they are killed and the hippocampus was removed for biochemistry analyses. The bigger particle size impaired spatial memory with significant decrease on BDNF neurotrophin, but, showed high antioxidant capacity and no injured membrane lipid. This deleterious action on memory that may be attributed to high antioxidant capacity, since an amount of ROS is necessary for cell signalization and thereby memory consolidation. These results are very interesting, because, the same nanomaterial in different particle size demonstrated different action on brain tissue.

Keywords: Fullerene nC_{60}, spatial memory, BDNF, oxidative stress, rats hippocampus.

1. Introduction

Nowadays, nanotechnology is subject to fast development; consequently, the potential hazards and environmental impacts of nanomaterials, such as nanoparticules, are receiving great attention [1]. The nanoparticules possess at least one dimension in the 1 to 100- nm range [2]. In this study we use two different particle sizes of 0,20 and 0,45 µm to analyze the possible differences between in biological terms.

This large expansion of nanotechnology generates a new study area, the nanotoxicology, which aims to analyze the effects of nanometer size particles in the organisms [3]. A specific nanocompound, Buckminsterfullerene (for short, fullerene $C_{60,}$ nanoC_{60} or nC_{60}), was characterized in 1986 by Kroto and coworkers. Since of this, other study are being development to analyze interactions of these nanoparticules with biological systems [4].

Although the physical and chemical characteristics of these nanocompounds are well known [5], few studies have shown the interaction of nC_{60} with biological systems correlating biochemical responses concomitantly with behavior and memory.

Bosi et al. [6] suggested that fullerenes and carbon nanomaterials are promising compounds in the development of therapeutic methods for the treatment of neurodegenerative disorders, such as Parkinson's and Alzheimer's diseases. In these diseases there is overproduction of oxygen and nitric oxide reactive species that probably stimulate the over-excitation of glutamate receptors [7,8] augmenting the excitotoxicity. Oxidative stress is known to induce cellular instability by a cascade of events that can lead to programmed cell death [9,10].

Studies with nC_{60} have showed conflicting results. For example, antioxidant characteristics of fullerenes and derivatives are based on their capability to act like radical sponges to reactive oxygen species (ROS). Xiao et. al. [11] observed a decrease in the ROS concentration after nC_{60} exposure and this nanocompound seem to actuate as a potent antioxidant, protecting human skin keratinocytes cells against oxidative stress through 'scavenging' capacity. In the other hand, Sayes et. al. [12] found that nC_{60} has significant toxicity in fibroblasts, hepatocytes and astrocytes cell culture and induced lipid peroxidation suggesting an oxidative stress situation.

Kamat et al. [13], was a pioneer in the study with nC_{60}, because showed another characteristic of this compound. In this study, this nanocompound induced oxidative stress through photoexcitation. When nC_{60} was incorporated in rat liver microssomes in the form of cyclodextrin complex and exposed to UV or visible light was induced significant oxidative damage in terms of lipid peroxidation and protein damage. The nC_{60} action in the brain of mammals in the presence or absence of light is so far unknown. However, some aspects should be considered, for example if nC_{60} can be deleterious to brain tissue in terms of learning, memory spatial and oxidative damage.

Memory is an ability of organisms to store, retain, and recall information and experiences, being those physiological conditions necessary to survival, and in the interaction with environment [14-16]. Besides, is a good parameter to analyze the brain condition [17]

Brain-derived neurotrophic factor (BDNF) has an important action in mammalian brain [18]. The BDNF, a small dimeric secretory protein, is an important neurotrophic factor critically involved in regulating of the survival and differentiation of neuronal populations in the peripheral and central nervous systems during development and in the adult brain [18]; and it has important roles in excitatory synaptic transmission and plasticity [19].

The ROS production is a physiological process essential for the functioning of the nervous system [20]. However, an imbalance between antioxidant defenses and the intracellular ROS concentration can trigger oxidative stress being that this situation can also be characterized by signaling pathway disruptions [21]. Besides, it is known that ROS have an important role in neurodegeneration and cognitive decline, such as Alzheimer Disease (AD), where post mortem human studies showed a higher lipid peroxidation levels in the brain of AD patients compared with controls [22]. On the other hand, certain ROS production is necessary to cellular signaling [23]. For example, H_2O_2 is nowadays considered as secondary messenger, necessary to synaptic plasticity [24]

The nervous tissue is very sensitive to oxidative damage because it has a high metabolic activity with elevated oxygen consumption (35 mL/min/kg). This organ has high amounts of polyunsaturated fatty acids (PUFAs) in cellular membrane that are susceptive to lipid peroxidation [25-27]. Besides, a significant

amount of transition metals with redox activity, like iron, and low level of antioxidant capacity in specific brain zones like hippocampus contribute to vulnerability of brain to oxidant substances [28]. All these characteristics turn the brain into an excellent scenario to the induction of oxidative stress.

So, this paper aims to analyze the action of the water-soluble nC_{60} in hippocampus of rats, through the analysis of behavioral parameters, molecular BDNF levels and oxidative stress, using the Wistar rat model.

2. Material and Methods

2.1. Preparation of fullerene suspension

An aqueous Fullerene (C_{60}) suspension (200mg/L; SES Research, 99% purity) was stirred in Milli Q water for two months under constant artificial white light. After that the suspension was centrifuged by one hour in 15ºC with 25000 x g. Then the suspension was filtered sequentially trough 0,45 and 0,20 µm filters. The carbon concentration was estimated by measuring total carbon concentration with a TOC-V CPH (Shimadzu) total carbon analyzer. Later, the new suspensions were characterized with transmission electronic microscopy. (Figure 1)

2.2. Animals models

Males Wistar rats (Rattus norvegicus, n= 50), young of 2-3 months of age were obtained from our own breeding colony, Universidade Federal de Rio Grande (Rio Grande, RS, Brazil). They were housed five per cage, under 12 h light/dark cycles, control temperature around 21 ±1º C, food and water *ad libitum.* All animal procedures were in accordance with Animal Care and Use Committee of the Brazilian College of Animal Experiments, and all efforts were made to reduce the number of used animals and their suffering.

2.3. Surgery and infusion procedures

Male Wistar rats were anesthetized under deep ketamine and xilasine. A guide cannulae were implanted in the dorsal CA1 region using coordinates (A −4.3, L ±3.0, V 1.8) taken from the atlas of

Paxinos and Watson [29]. Cannulae were fixed to the skull with dental acrylic [30]. After (48 h) recovery from the surgery, these animals were trained in Water Maze (WM) task by five days and tested in sixth day. Infusions were given 0 min after each training session through 27-gauge infusions cannulae fitted into the guides. Through the infusion cannulae rats received 0 min after the training a bilateral administration of saline in the control group and nC_{60} 0,20µm and 0,45µm on the treatment groups. The tip of the infusion cannula protruded 1.0 mm beyond the guide cannula; by this procedure the drug reaches the CA1 hippocampus region. In all groups infusions had a volume of 1 µL.

2.4. Behavioral parameters

A modification of the spatial version described by Morris (1984) was used. Data was obtained through a tracking video system (EthoVision®, NOLDUS). The Water Maze (WM) consisted of a circular dark tank (168 cm diameter and 70 cm depth) filled with water. A hidden platform was fixed in one of the four virtual quadrants for all of the training sessions. Visual cues were placed in the WM room. The water was kept at 24±1 °C. On the first day, the rats faced a 4 trial training session of 120 s with a 70 s interval. For each trial, the animals started from a different position in the WM. In the end of each session (0 minutes post training) the rats were infused with 1uL of saline (CTR) or nC_{60} 0,2 µm (1.10^{-5}mg/side) or 0,45 µm (1.10^{-5}mg/side). In the training sessions, learning progress was evaluated as the latency to reach the hidden platform (escape). In the retention test day the platform was removed and the animals swam freely once for 90s on the all the tank and to observe the memory retention was analyzed the time spend over the previous location of the escape platform. After behavioral task, the rats were decapitated and hippocampus were immediately dissected and stored at – 80ºC.

2.5. Biochemistry assays

2.5.1 Elisa method to detected BDNF

Tissues were homogenized with lysis buffer, centrifuged and the supernatant was separated. ELISA is a highly sensitive method for measuring low levels of neurotrophin. It is necessary dilute the supernatant

sample by adding 4 volumes of Dulbecco's Phosphate Buffered Saline (DPBS). First step is plate coating using a multichannel pipettor to add 100µl to each well of a polystyrene ELISA plate, than has coating antibody, seal the wells with a plate sealer, and incubate without shaking overnight at 4°C. Add the dilute sample and then 100µl Anti-Human BDNF pAb, incubate for 2 hours with shaking in room temperature and next accurately add 50µl of Anti-IgY HRP and incubate for 1 hour with shaking to and room temperature. After that put 100µl of room-temperature TMB One Solution for wash to each well for 10 minutes with shaking and then add 100µl/well HCl 1N to stop reaction and record the absorbance at 450nm on a plate reader.

2.5.2. Reactive oxygen species (ROS) measurement

Immediately after dissection, brain samples were homogenized (1:5 w/v) in cold buffer (40 mM Tris-HCl)- pH 7,4) and centrifuged. The supernatants were used for determination of ROS using 2',7'-dichlrofluorescein-diacetate (H_2DCF-DA, Sigma USA) which generates a fluorochrome detected at 488/525 nm for excitation/emission wavelengths [31]. Readings were performed with a fluorescence microplate reader (Perkin Elmer Victor 2 fluorescence, USA).

2.5.3. Determination of antioxidant capacities against peroxyl radicals

The antioxidant capacity against peroxyl radicals was measured according to the method of Amado et al [32]. Using a fluorescence microplate reader (Victor 2, Perkin Elmer), at a programmed temperature of 35 ^{o}C, at which peroxyl radicals were produced by thermal decomposition of ABAP (2,2′-azobis 2 methylpropionamidine dihydrochloride) [33]. Immediately before the reading, 10 µL of the fluorescent probe 2´,7´ dichlorofluorescein diacetate (H_2DCF-DA) were added to each well at a final concentration of 40 µM [31]. H_2DCF-DA is cleaved by esterases present in the samples and the non-fluorescent compound 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.

2.5.4. Lipid peroxides content

Lipid peroxidation was quantified by the thiobarbituric acid reactive substances (TBARS) method, according to Oakes and Kraak [34], measures the oxidative damage to lipids. The fluorescence measured at 515nm to excitation and 553nm to emission wavelengths. The oxidative damage to lipids was expressed as nmols of equivalents of TMP (tetramethoxypropano, standard reaction) per mg of tissue, based in the TMP curve.

2.5.5. Determination of Reduced Glutatione (GSH) concentration and Glutamante Cystein Ligase (GCL) activity

Reduced glutathione (GSH) was analyzed according to [35], from the reaction of naphthalene dicarboxialdehyde (NDA) with GSH or γ-glutamylcysteine (γ-GC) to form highly fluorescent cyclized products detected on a fluorescence microplate reader (Victor 2, Perkin Elmer) at 472 /528 excitation/emission wavelenghts.

Total protein content was analyzed using a commercial kit (Doles Ltda, Brazil) based on the Biuret method. Protein and enzymes determinations were performed at least in triplicate.

2.6. Statistical analyses

Statistical differences between various parameters were tested through analysis of variance (ANOVA), followed by Newman-Keuls test ($\alpha = 0.05$). Assumptions of normality and variance homogeneity were previously checked and mathematical transformation applied if necessary [36].

3. Results

A significant difference in the escape latency among 0,45 µm and the 0,20 µm and control groups were found in the learning phase of this task ($p<0.05$) (**Figure 2a**), showed a impairment in learning in group with 0,45 µm treatment. In the retention test, the time of permanency in the quadrant target (TQ) was influenced by nC_{60} 0,45 µm treatment (**Figure 2b**).

The BDNF levels was significant diminished ($p<0.05$) in the hippocampus in the group exposed to 0,45 µm particles size of nC_{60} (**Figure 3**).

There was a significant decrease in ROS concentration ($p<0.05$) in the group exposed to 0,45 µm particles size of nC_{60} when compared with other groups (**Figure 4a**).

Total antioxidant capacity against peroxyl radicals was augmented ($p<0.05$) in both treatment groups when compared to control group (**Figure 4b**).

Any significant changes in terms of TBARS content was observed ($p> 0.05$) in all groups submitted to experimental conditions (**Figure 5**).

GCL activity and GSH levels were not affected after exposure to both particle size of nC_{60} ($p> 0.05$) (**Figure 6a** and **6b** respectively).

4. Discussion

In this study was observed that Nano-C_{60} has impairment in the Water Maze task performance. In the start of experiment all groups showed the same escape latency to found the platform however, how expected, in the second day all of groups decreased escape latency in finding the platform. Analyzing the behavior in the group with 0,45µm sized particles, was observed a raise in the escape latency to find the hidden platform from the third day on, with significant difference when compared with the group with 0,20 µm sized particles and the control group. The same condition was observed on the testing day, when the 0,45µm group did not perform well concerning their orientation in the dark thank. This could be inferred as they spent less time swimming in the target quadrant (TQ) than other groups. These results suggest that the nC60 with bigger particle size can influence on the spatial memory consolidation ability.

As mentioned in the **section introduction**, the BDNF is an important neurotrophin [37]. BDNF plays a crucial role in learning and memory being that highest levels of BDNF expression in the mammalian brain is observed in the hippocampus [38], therefore BDNF has a particularly important role on hippocampus-dependent learning as spatial memory. In fact, BDNF expression is decreased in the

hippocampus of Alzheimer's disease patients suggesting the importance of this neurotrophin in the integrity of normal brain functions [39].

In this study, was observed significant decrease ($p<0.05$) in amount of this neurotrophin in the group exposed to 0,45 µm of nC_{60}. Concomitantly we observed in the same group a decrease in learning and spatial memory. These results suggest the link between lower BDNF levels and impairment of learning and spatial memory. In fact, [40] showed that when BDNF was supressed, the animals demonstrated difficult in the memory formation.

One hypothesis to decrease BDNF levels can be related to glutamatergics receptors. Jin et. al. [41] suggests fullerenols, that are C_{60} derivatives, exert neuroprotective functions blocking glutamate receptors and lowering the intracellular calcium on the brain tissue. In fact, the fullerenol is as antagonist substance of NMDA and AMPA receptors, probably causing considerable decrease in neuronal signaling. Moreover, Mokrushin [42], found a 40% decrease in the amplitude of the excitatory postsynaptic potential (EPSP) in AMPA and NMDA components after n-C_{60} treatment in olfactory cortex slices.

BDNF decrease synthesis may be involved in the role of these glutamatergic receptors to induce early gene transcription [43] According with this hypothesis our results of ELISA showed lower quantity of neurotrophin. So, is probably that nC_{60} also can block these receptors resulting in BDNF decrease.

Interestingly, between treatment groups we observed a significant influence on the action of the different particle sizes. In relation to learning, spatial memory and the hippocampal BDNF, the small particle (0,2 µm of nC_{60}) did not show any changes when compared with other groups. Thus, based in our results the small sized particles seems are less hazardous. Perhaps minor particles can be ideal for the biomedical neuronal area, for example, to carrying drugs. However, more studies are necessary to confirm this hypothesis.

Reduced glutathione (GSH) is considered an important cellular antioxidant being effective against reactive oxygen species (ROS) and/or oxidative damage [44]. This antioxidant can be modulated in response to several chemical substances exposure. In fact, Oberdörster [45] showed a depletion of GSH levels in gills of Juvenile Largemouth Bass when exposed to nC60 on the water, so the GSH was used as an

indication of oxyradical scavenging ability, showing that the antioxidant defense system is overwhelmed by oxidative scenarious. On the other hand, Bickley and McClellan-Green observed an increase in GSH levels in gills of *Fundulus heteroclitus* larvae stage after nC_{60} exposure [46].

In mammals cells the GSH levels also seem be modulated after nC_{60} exposure. Sayes [12] showed increases in GSH concentration in HDF, HepG2, and NHA cells after nC_{60} exposure suggesting that increase in the reduced glutathione synthesis in the cells can be response to oxidative stress caused by fullerene. Interestingly in this study, any changes in terms of GSH levels in the brain were observed after nC_{60} exposure in different particles sizes. The limiting step rate of GSH synthesis is due to activity of glutamate cysteine ligase (GCL). In this study we analyzed the GCL activity and not we observed any significant changes, this result according with GSH levels observed in our study.

In this study we observed a decrease in Reactive Oxygen Species (ROS) concentration in rat hippocampus treated with fullerene 0,45 µm. In the scientific literature some question are still unclear about nC_{60} real action. Some studies suggest that fullerenes possess reducing properties as antioxidant potential similar to quinone and vitamin E. [47]. While Arbogast, observed that nC_{60} and its derivatives can induce ROS production under irradiation condition [48]. Hotze, suggest that photosensitized molecules like nC_{60} are capable of transferring light energy to chemical oxidation potential in the form of ROS [49]. In this study we made to nC_{60} exposure without light presence. However, the size particle seem has bigger influence in scavenging action observed here, once both groups exposed to nC_{60} (0.2 and 0.45 µm) were submitted the same experimental conditions and only group exposed to 0,45 µm showed significant decrease in the ROS concentration.

Moreover the GSH levels and GCL activity analyzed in this study not showed any changes after nC_{60} exposure, the total antioxidant capacity against peroxyl radicals demonstrated a significant increase in both groups treated with either nC_{60}. This result suggested that the nC_{60} *per se* can be actuating as antioxidant once that this nanocompound possess characteristics of like radical sponges [11].

Ali et. al. [50], observed that nC_{60} and its derivatives, especially water-soluble derivatives, are potent antioxidant in mammalian cells against oxidative stress through of scavenging capacity. Gharbi et.

al. [51] showed that nC_{60} act as antioxidant in liver of rats diminishing the injury induced by carbon tetrachloride (CCl_4). These results are similar with our dates where the nC_{60} actuated as antioxidant substance.

Sayes et al. [12] observed that nC_{60} can induce oxidative damage in lipids in human dermal fibroblast, human liver carcinoma cells and neuronal human astrocytes. So, suggesting that in this case the nC_{60} induced an oxidative stress condition and this situation was completely reverted by presence of antioxidant *L*-ascorbic acid. A similar result also observed in brain of Juvenile Largemouth Bass where the nC_{60} induced an increase in lipid peroxidation (LPO) levels [45], in fact the brain is an organ susceptible to oxidative damage because is rich in polyunsaturated fatty acid and low antioxidant defense. A different result was observed in our study where any lipid oxidation was observed after nC_{60} exposure in both groups.

As previously mentionate nC_{60} can absorb ultraviolet light and then generate ROS affecting amino acids, nucleic acids and the carbon double bonds of membrane phospholipids inducing cell damage and death [52]. According to Kamat et. al. [53], fullerene became oxidant substance in light presence. In this study, our nC_{60} suspension was infused directly into the hippocampus; therefore, protected from the ultraviolet and visible light, possibly due to this was not observ ed lipid peroxidation in our samples.

Thus, in present study we observed an antioxidant action of nC_{60} and also amnesic effect in the groups exposed to bigger particle size. These results are interesting because in this case, in the same time that nC_{60} showed antioxidant characteristics was also observed an amnesic effect demonstrated by impairment of the learning and spatial memory. These hypotheses can be explained by capacity of nC_{60} to eliminate both radical superoxide anion and H_2O_2 [54]. It is know that memory formation needs a certain amount of ROS to start the cellular signalization; mainly H_2O_2 that currently is being considered as a second messenger on synaptic plasticity [Serrano e klan]. On the other hand, kamsler and Seagel [55] observed that in transgenic mice overexpressing superoxide dismutase (SOD) there was impairment in their ability to express hippocampal long-term potential (LTP) necessary to memory formation [19]. In the same study the addition H_2O_2 in the wild mice also was observed impairment on the LTP.

In summary, a certain quantity of ROS, mainly H_2O_2, is necessary to trigger the signaling neuronal event that result in LTP; being indispensable to memory consolidation. However, a high quantity of ROS can cause cellular injury and also to affect the synaptic plasticity, for this reason some studies has suggested that H_2O_2 play role paradoxical [24,55]. A lower ROS concentration seem to decrease the capacity of learning and spatial memory, in fact in this study we observed that in hippocampus exposed to 0,45 µm of nC_{60} a decline in ROS concentration concomitantly with amnesic effect.

Acknowledgments: Ândrea Kraemer is graduate fellow from the Brazilian agency CAPES. Juliane Ventura Lima receives a post-doctoral fellow from Brazilian agency CAPES (Ciências do Mar Nº 09/2009). Gustavo Parfitt and Daiane Acosta received a graduate fellowship from the Brazilian agency CNPQ. Daniela Martí Barros and José M. Monserrat are research fellows from CNPQ. The research was supported by a grant from CNPQ 577159/2008-9.

5. References

1. Zhu X, Zhu L, Land Y, Chen Y. Oxidative stress and growth inhibition in the freshwater fish Carassius auratus induced by chronic exposure to sublethal fullerene aggregates. Environ Toxicol Chem 2008; 27: 1979-1985.

2. Garnett MC and Kallinteri P, Nanomedicine and nanotoxicology some physiological principles. Occupational Medicine 2006; 56: 307-311.

3. Suh WH, Suslick KS, Stucky GD, and Suh Y. Progress in Neurobiology. Progress in Neurobiology 2009; 87: 133-170.

4. Nielsen GD, Roursgaard M, Jensen KA, & Poulsen SS. In vivo. *Pharmacology & Toxicology 2008*; 197-208.

5. Johnston HJ, Hutchison GR, Christensen FM, Stone V, and Safety E. REVIEW The Biological Mechanisms and Physicochemical Characteristics Responsible for Driving Fullerene Toxicity. Toxicological Sciences 2010; 114: 162-182.

6. Bosi S, Spalluto G, and Prato M. Fullerene derivatives: an attractive tool for biological applications. European Journal of Medicinal Chemistry 2003; 38: 913-923.

7. Cassarino DS, Fall CP, Swerdlow RH, Smith TS, Halvorsen EM, Miller SW, and Parks JP. Elevated reactive oxygen species and antioxidant enzyme activities in animal and cellular models of Parkinson's disease. Science 1997; 77-86.

8. Mattson MP and Goodman Y . Different arrlyloidogenic peptides share a similar mechanism of neurotoxicity involving reactive oxygen species and calcium. Science 1995; 676: 219-224.

9. Misirkic MS, Todorovic-markovic BM, Vucicevic LM, Janjetovic KD, Jokanovic VR, Dramicanin MD, Markovic ZM, and Trajkovic VS. The protection of cells from nitric oxide-mediated apoptotic death by mechanochemically synthesized fullerene (C60) nanoparticles. Cultures 2009; 30: 2319-2328.

10. Circu ML and Aw TY. Reactive oxygen species, cellular redox systems, and apoptosis. Free Radical Biology & Medicine 2010; Matrix 48: 749-762.

11. Xiao L, Takada H, Maeda K, Haramoto M, and Miwa, N.Antioxidant effects of water-soluble fullerene derivatives against ultraviolet ray or peroxylipid through their action of scavenging the reactive oxygen species in human skin keratinocytes. Biomed. Pharmacother 2005; 59, 351-358.

12. Sayes CM, Gobin AM, Ausman K.D, Mendez J, West JL, Colvin VL. Nano-C60 cytotoxicity is due to lipid peroxidation. Biomaterials 2005; 26: 7587-7595.

13. Kamat JP, Devasagayam TP., Priyadarsini KI, Mohan H, Mittal J P. Oxidative damage induced by the fullerene C 60 on photosensitization in rat liver microsomes. Chemico-Biological Interactions 1998; 114: 145 - 159.

14. Izquierdo I and Medina JH. Memory formation: the sequence of biochemical events in the hippocampus and its connection to activity in other brain structures. Neurobiology of learning and memory 1997; 68: 285-316.
15. Kandel ER. The molecular biology of memory storage: a dialogue between genes and synapses. Science 2001; 294: 1030-8.
16. McGaugh JL. Memory--a Century of Consolidation. Science 2000; 287: 248-251.
17. Dorman DC. An Integrative Approach to Neurotoxicology. Toxicologic Pathology 2000; 28: 37-42.
18. Soulé J, Bramham CR. Brain-derived neurotrophic factor and control of synaptic consolidation in the adult brain . Biochemical Society Transactions 2006: 34; 600-604.
19. Bramham CR, and Messaoudi E. BDNF function in adult synaptic plasticity : The synaptic consolidation hypothesis. Progress in Neurobiology 2005; 76: 99-125.
20. Halliwell B. Biochemistry of Oxidative Stress. Biochemical Society Transactions 2007;vol. 5, part 5.
21. Jones DP. Redefining Oxidative Stress. Antioxidants e redox signaling 2006; 8: 9 & 10.
22. Praticò D. Evidence of Oxidative Stress in Alzheimer's Disease Brain and Antioxidant Therapy. New York 2008;78: 70-78.
23. Serrano F and Klan E. Reactive oxygen species and synaptic plasticity in the aging hippocampus. Aging Res Rev 2004; 431-443.
24. Kamsler A & Segal M. Hydrogen Peroxide as a Diffusible Signal Molecule in Synaptic Plasticity. *Molecular Neurobiology 2004;* **29:** 167-178.
25. Packer L, Tritschler HJ, Wessel K. Neuroprotection by the metabolic antioxidant □-lipoic acid. Free Radical Biology and Medicine 1997; 22: 359-378.

26. Cardozo-Pelaez F, Brooks PJ, Stedeford T., Song S., Sanchez-Ramos J. DNA damage, repair, and antioxidant systems in the brain region: a correlative study. Free Radical Biology and Medicine 2000; 28: 779-785.

27. Qiao D, Seidler FJ, Slotkin TA. Oxidative mechanisms contributing to the developmental neurotoxicity of nicotine and chlorpyrifos. Toxicol. Appl. Pharmacol., 2006: 17-26, 2005

28. Balu M, Sangeetha P , Haripriya D, Panneerselvam C. Rejuvenation of the antioxidant system in the central nervous system of aged rats by grape seed extract. Neuroscience Letters 2005; 383: 295-300.

29. Paxinos G, Watson C. (1986). The Rat Brain in Stereotaxic Coordinates.

30. Barros DM, Ramirez MR, dos Reis EA, and Izquierdo I. Participation of hippocampal nicotinic receptors in acquisition, consolidation and retrieval of memory for one trial inhibitory avoidance in rats.Neurocscience 2004;126: 651- 656.

31. Ferreira-Cravo M, Reinhardt Piedras F, Moraes TB, Ferreira JLR, Salomão DPF, Dornelles Machado M, Geracitano LA, Monserrat JM. Antioxidant responses and reactive oxygen species generation in different body regions of the estuarine polychaeta Laeonereis acuta (Nereididae). Chemosphere, 2007;66: 1367–1374

32. Amado LL, Longaray Garcia M, Ramos PB, Freitas RF, Zafalon B, Ribas Ferreira JL, Yunes JS, Monserrat JM. A method to measure total antioxidant capacity against peroxyl radicals in aquatic organisms: application to evaluate microcystins toxicity. Sci. Total Environ 2009; 407: 2115–2123

33. Wiston GW, Regoli R, Dugas AJ, Fong JH, Blanchard. A radid gas chromatographic assay for determining oxyradical scavenging capacity of antioxidants and biological fluids. Free Rad. Biol. Med. 1998; 24:480-493

34. Oakes KD, Kraak GJV. Utility of the TBARS assay in detecting oxidative stress in white sucker (Catostomus commersoni) populations exposed to pulpmill effluent. Aquat. Toxicol 2003; 63: 447–460.

35. White CC, Viernes H, Krejsa CM, Botta D, Kavanagh TJ. Fluorescence-based microtiter plate assay for glutamate-cysteine ligase activity. Anal. Biochem. 318, 175-180, 2003.

36. Zar J H. Biostatistical analysis. Ed. Prentice Hall, 2da ed., New Jersey. 718 pp. 1984.

37. Bekinschtein P, Katche C, Slipczuk L, Rossato JI, Goldin A, Izquierdo I, and Medina JH BDNF is essential to promote persistence of long-term memory storage. Sciences-New York 2007; 0-5.

38. Murer MG, Yan Q, Raisman-Vozari R. Brain-derived neurotrophic factor in the control human brain, and in Alzheimer's disease and Parkinson's disease. Prog Neurobiol 2001; 63(1):71-124.

39. Connor B, Dragunow M.. The role of neuronal growth factors in neurodegenerative disorders of the human brain. Brain Res Rev1998; 27:1–39

40. Heldt SA, Stanek L, Chhatwal JP and Ressler KJ. Hippocampus-specific deletion of BDNF in adult mice impairs spatial memory and extinction of aversive memoriesSpecific BDNF deletion in adult hippocampus. Molecular Psychiatry 2007; **12**: 656-670.

41. Jin H, Chen WQ, Tang XW, Chiang LY, Yang CY, Schloss JV, Wu JY. Polyhydroxylated C 60 , Fullerenols, as Glutamate Receptor Antagonists and Neuroprotective Agents. Journal of Neuroscience Research 2000; 607: 600 - 607.

42. Mokrushin AA. Neurotoxic Effects of Fullerenes on the Electrical Activity of Surviving Sections of the Rat Brain Olfactory Cortex. Doklady Biological Science 2001; 377122-124

43. Halliwell B, Gutteridge, JM. Free radicals in biology and medicine. Oxford University Press,2007; 851.

44. Oberdörster E. Manufactured Nanomaterials (Fullerenes, C 60) Induce Oxidative Stress in the Brain of Juvenile Largemouth Bass. Environmental Health Perpectives 2004;112: 1058-1062.

45. Bickley TM and McClellan-Green P,Toxicity of Aqueous Fullerene in Adult and Larval Fundulus Heteroclitus. Nanomaterials in the Environment 2008; 27: 9 1694-1971.

46. Martin N, Sánchez L, Illescas B, Pérez I. C60 -Based Electroactive Organofullerenes. Chemical Review 1998; 98: 2527-2547.

47. Arbogast JW, Darmanyan AP, Foote CS, Rubin Y, Diederich FN, Alvarez MM, Anz SJ, Whetten, R. L. REVISTA 1991; Society, 11-12.

48. Hotze, EM, Labille, J, Alvarez, P, and Wiesner, MR. Mechanisms of photochemistry and reactive oxygen production by fullerene suspensions in water. Environmental science & technology 2008: 42; 4175-80.

49. Ali SS, Hardt JI, Quick KL, Kim-Han JS, Erlanger BF, Huang TT, et al. Abiologically effective fullerene (C60) derivative with superoxide dis- mutasemimeticproperties. Free Radic Biol Med 2004;37(8):1191–202.

50. Gharbi N, Pressac M, Hadchouel M, Szwarc H, Wilson SR, Moussa F. [60]Fullerene is a Powerful Antioxidant in Vivo with No Acute or Subacute Toxicity. Nano Letters 2005; 5: 12, 2578-2585.

51. Jensen AW, Wilson SR, Schuster DI. Biological Applications of Fullerenes. Science 1996; 4: 767-779.

52. Kamat JP, Priyadarsini KI, Mohan H. Reactive oxygen species mediated membrane damage induced by fullerene derivatives and its possible biological implications. Toxicology 2000; 155: 55 - 61.

53. Dugan LL, Lovett EG, Quick KL, Lotharius J, Lin TT, Malley KL. Fullerene-based antioxidants and neurodegenerative disorders. Parkinsonism & Related Disorders 2001; 7: 1-4.

54. Kamsler A and Segal M. Paradoxical Actions of Hydrogen Peroxide on Long-Term Potentiation in Transgenic Superoxide Dismutase-1 Mice. The Jounal of Neuroscience 2003; 23: 10359 -10367.

Figure Captions

Figure 1. The picture of the Fullerene 0,20µm Suspension aggregates (TEM micrograph).

Figure 2. Intrahippocampal infusions of 0,20 and 0,45 µm of nC_{60} immediately after the training in WM (a) Training section during the 5 days in Water Maze task (express in mean and SEM of escape latency in seconds). (b) Test section in water maze task (express in mean and SEM of percentage of time spent in the target quadrant). Different letters indicate significant differences ($p<0.05$) between means of different treatments. Data are expressed as mean ±1 standard error (n=6-7 per group).

Figure 3. BDNF levels (expressed by pg/mg of proteins). Different letters indicate significant differences ($p<0.05$) between means of different treatments. Data are expressed as mean ±1 standard error (n=3-4 group).

Figure 4. (a) Reactive oxygen species (ROS) concentration (area). **(b)** Antioxidant competence against peroxyl radicals (relative area). Different letters indicate significant differences ($p<0.05$) between means of different treatments. Data are expressed as mean ±1 standard error (n=3-4 per group).

Figure 5. TBARS content (nmol of MDA/mg of tissue). Different letters indicate significant differences ($p<0.05$) between means of different treatments. Data are expressed as mean ±1 standard error (n=4-5 per group).

Figure 6. (a) Glutamate cysteine ligase (GCL) activity (nM GSH/ mg of protein). **(b)** Glutathione (GSH) levels (nM/mg of protein). Different letter indicate significant differences ($p<0.05$) between means of different treatments. Data are expressed as mean ± 1 standard error (n= 4-5 per group).

Figures

Figure 1.

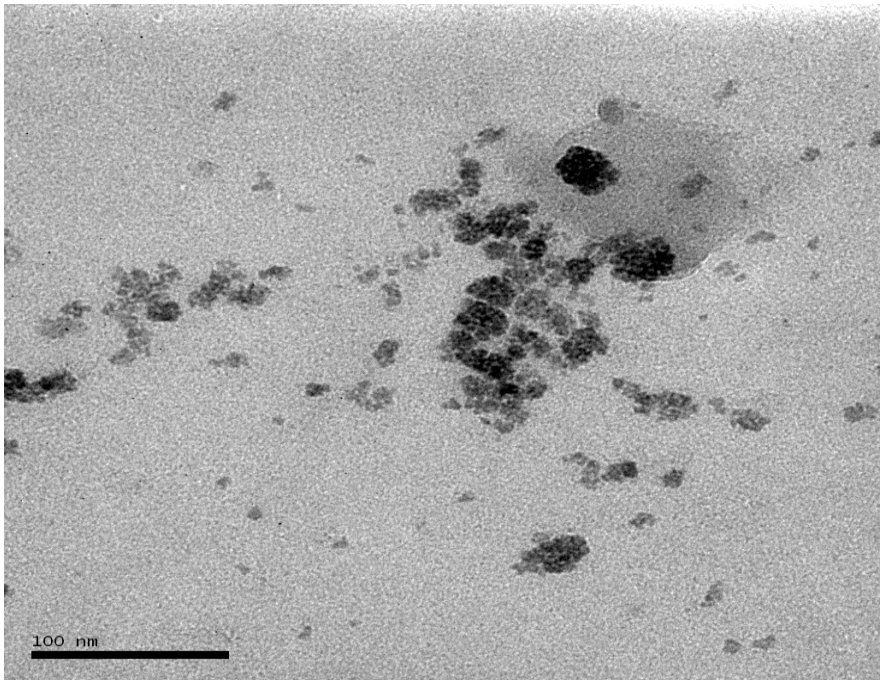
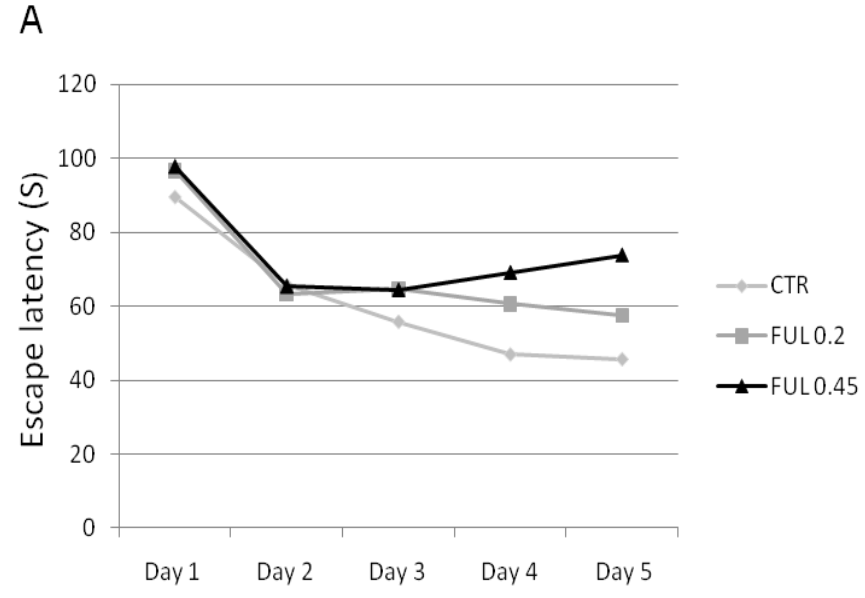


Figure 2.


A
120
100
80
60
40
20
0
Escape latency (S)
Day 1
Day 2
Day 3
Day 4
Day 5
CTR
FUL 0.2
FUL 0.45

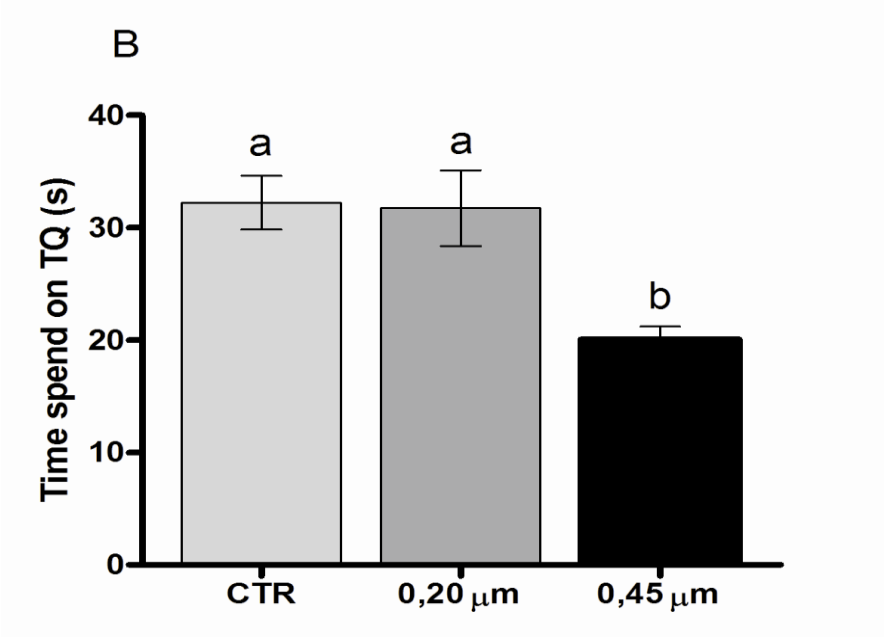

B
40
30
20
10
0
Time spend on TQ (s)
a
a
b
CTR
0,20 μm
0,45 μm

Figure 3.

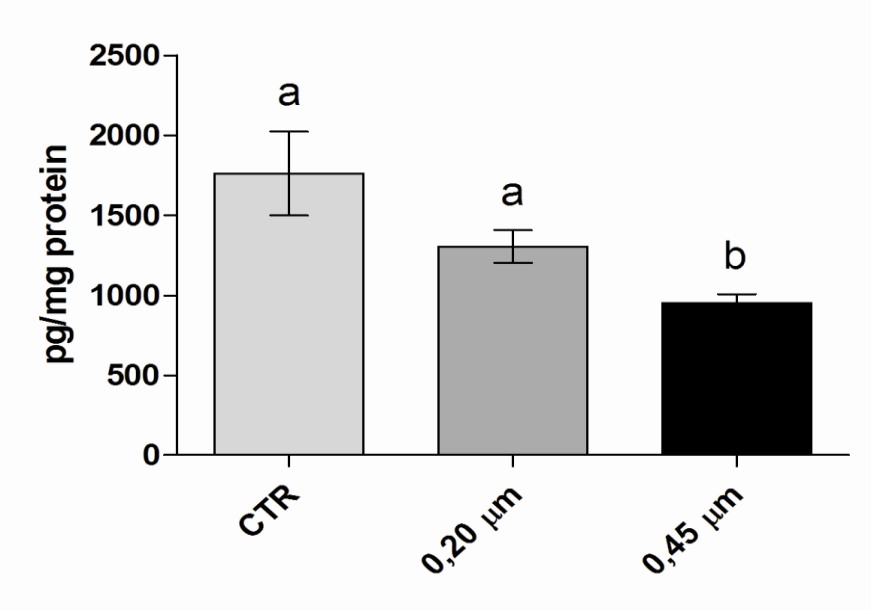
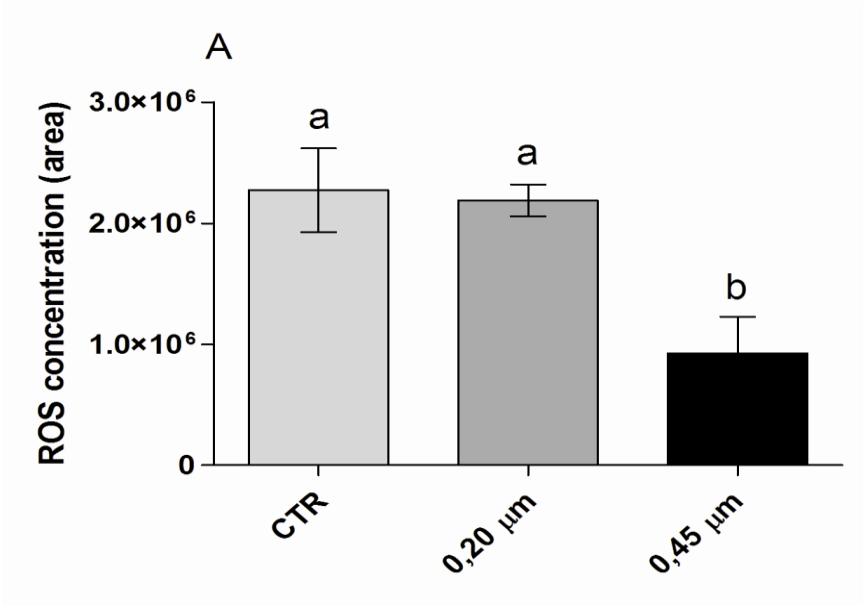


Figure 4.


A
ROS concentration (area)
3.0×10⁶
2.0×10⁶
1.0×10⁶
0
a
a
b
CTR
0,20 μm
0,45 μm

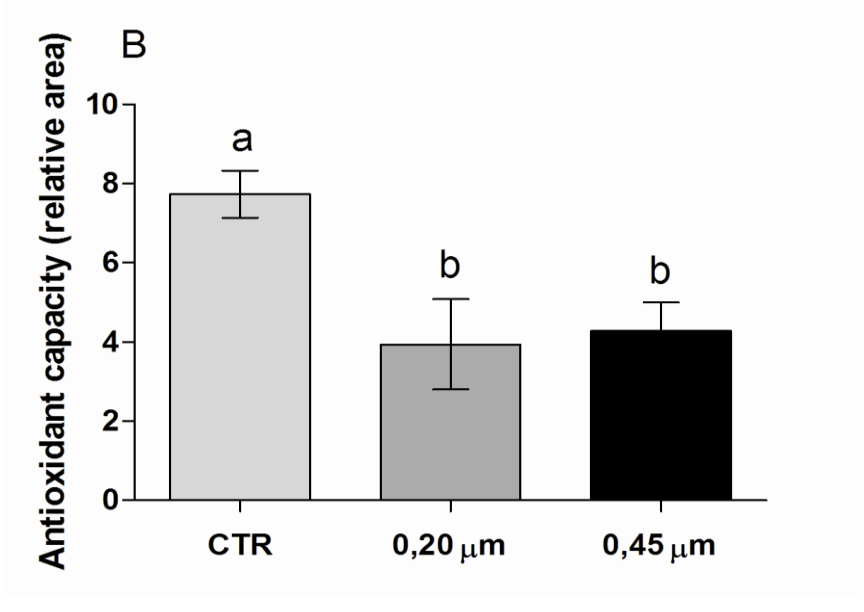

B
Antioxidant capacity (relative area)
10
8
6
4
2
0
a
b
b
CTR
0,20 μm
0,45 μm

Figure 5.

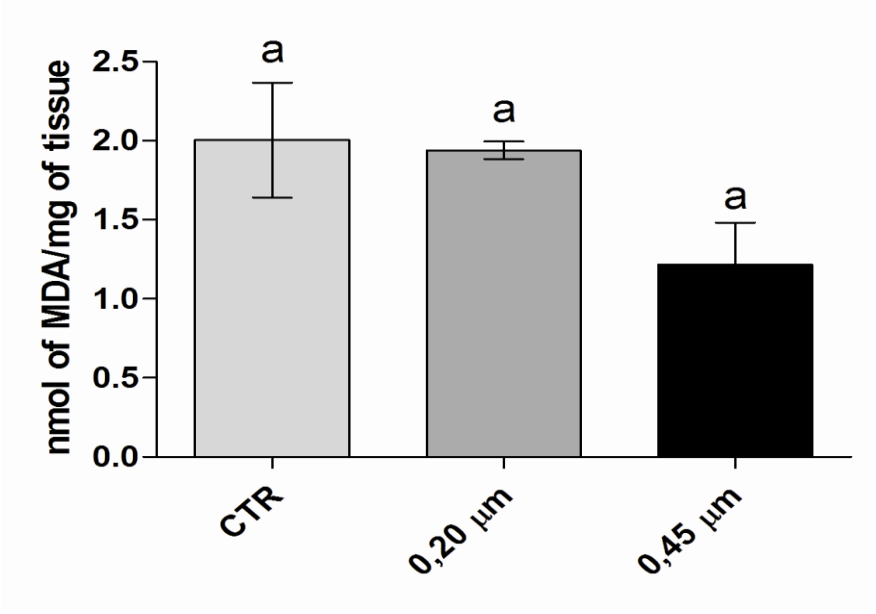
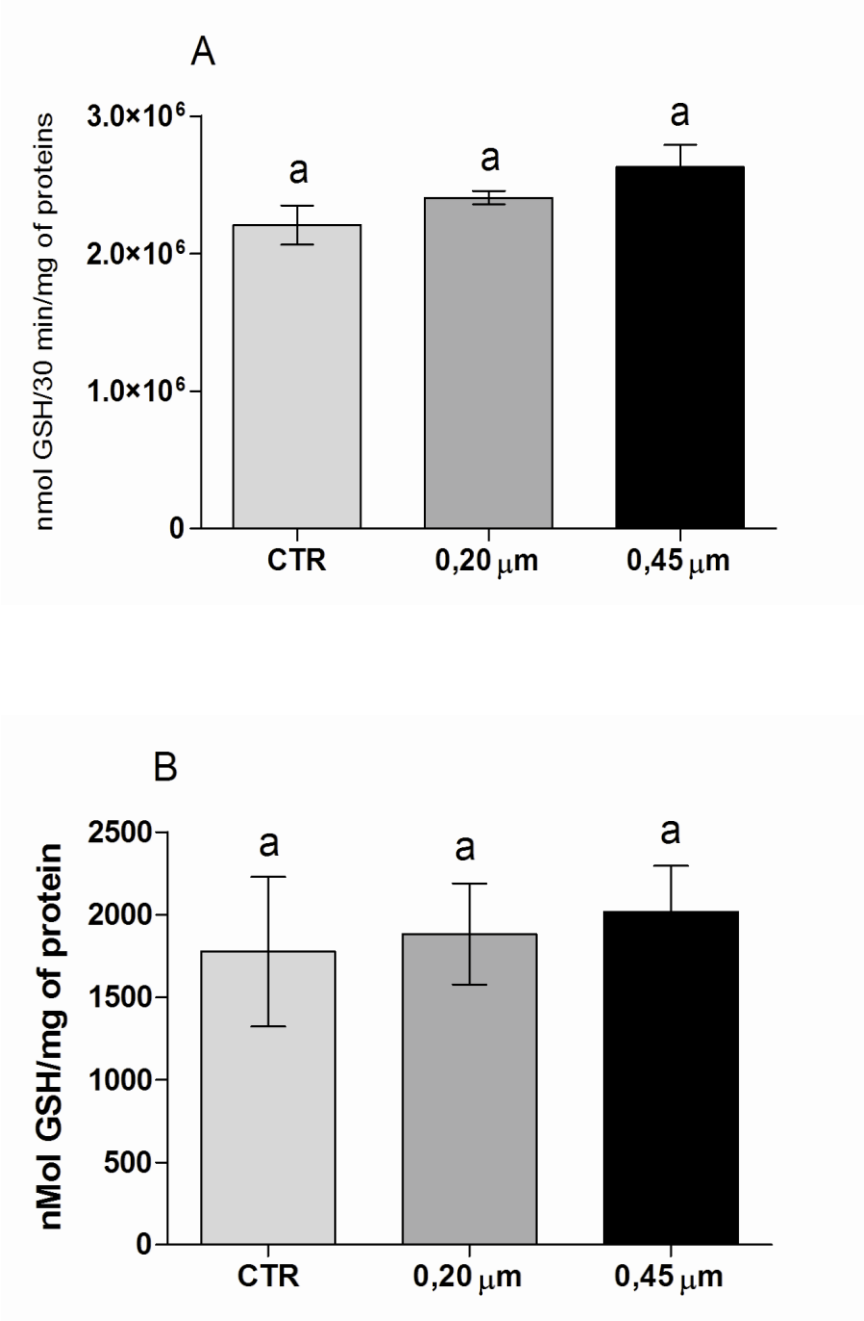


Figure 6.


A
nmol GSH/30 min/mg of proteins
3.0×10⁶
2.0×10⁶
1.0×10⁶
0
a
a
a
CTR
0,20 μm
0,45 μm
B
nMol GSH/mg of protein
2500
2000
1500
1000
500
0
a
a
a
CTR
0,20 μm
0,45 μm

Considerações finais

Em relação aos resultados obtidos com o nosso trabalho, o Fulereno teve uma ação amnésica nos animais tratados com as partículas de tamanhos maiores (0.45 μm) e também demonstrou ter uma ação antioxidante, uma vez que, a quantidade de espécies ativas de oxigênio (EAO) no hipocampo foi diminuída juntamente com o aumento da capacidade antioxidante total, sendo que, este mesmo tamanho de partícula não gerou peroxidação lipídica. Em relação ao sistema antioxidante endógeno (GCL e GSH), ele não se mostrou modulatório, talvez seja porque sua ação antioxidante deva estar relacionada na capacidade que o Fulereno tem de agir como uma esponja de radicais livres. Ao analisar os animais tratados com partículas de fulereno em tamanhos menores (0.20 μm), foi possível observar que não houve diferença significativa do controle em praticamente todas as análises, sendo somente diferente na capacidade antioxidante total, que se mostrou aumentada, igualando-se ao resultado encontrado nos animais tratados com as partículas de tamanho 0,45 μm.

Portanto, os resultados obtidos neste trabalho possibilitaram observar que o fulereno apresenta diferentes efeitos sobre o SNC relacionados com tamanho de partículas.

Bibliografia

1. Alves, O. L. Nanotecnologia, nanociência e nanomateriais: quando a distância entre presente e futuro não é apenas questão de tempo. Parcerias estratégicas 2004;18.
2. Thomas K., Aguar P, Kawasaki H, Morris J, Nakanishi J,Savage N. Research Strategies for Safety Evaluation of Nanomaterials, Part VIII: International Efforts to Develop Risk-Based Safety Evaluations for Nanomaterials. Toxicological Sciences 2006; 92: 23-32.
3. Nel A, Xia T, Mädler L, Li N. Toxic Potential of Materials at the Nanolevel. Science 2006; 311, 622.
4. Oberdörster G, Oberdörster E, Oberdörster J. Nanotoxicology: An Emerging Discipline Envolving from Studies of Ultrafine Particles. Enviromental Health Perpectives 2005; 113: 7 823-839.
5. Hirsch A, Brettreich M. Fullerenes Chemistry and Reactions 2005; 1º ed, wiley –VCH: Weinheim.
6. Echegoyen L, Echegoyen LE. Acc.Chem.Res 1998; 31: 593
7. Bosi S, Spalluto G, and Prato M. Fullerene derivatives: an attractive tool for biological applications. European Journal of Medicinal Chemistry 2003; 38: 913-923.
8. Izquierdo, I., and Medina, J. H. (1997). Memory formation: the sequence of biochemical events in the hippocampus and its connection to activity in other brain structures. Neurobiology of learning and memory 68, 285-316
9. Kandel, E. R. (2001). The molecular biology of memory storage: a dialogue between genes and synapses. Science (New York, N.Y.) 294, 1030-8.
10. McGaugh, J. L. (2000). Memory--a Century of Consolidation. Science 287, 248-251.
11. Dorman, D. C. (2000). An Integrative Approach to Neurotoxicology. Toxicologic Pathology 28, 37-42.

12. Sofroniew MV, Howe CL, Mobley WC. Nerve growth factor signaling, neuroprotection, and neural repair. Annu. Rev. Neurosci, 24: 1217-1281, 2001.

13. Huang EJ, Reichardt LF. Neurotrophins: roles in neuronal development and function. Annu Rev Neurosci, 24: 677-736, 2001

14. Lu B, Pang PT, Woo NH. The yin and yang of neurotrophin action. Nat. Rev. Neurosci. 6: 603-614, 2005.

15. Lipsky RH, Marini AN. Brain-derived neurotrophic factor in neuronal survival and behavior-related plasticity. Ann NY Acad Sci, 1122:130-143,2007

16. Alonso M, Vianna MRM,Delfino AM, Mello e Souza T, Pereira P, Szapiro G, Viola H, Pitossi F, Izquierdo I, Medina JH. BDNF-trigerred events in the rat hippocampus are required for both short- and long-term memory formation. Hippocampus, 12(4), 557-60, 2002.

17. Alonso M, Bekinschtein P, Cammarota M, Vianna MRM, Izquierdo I, Medina JH.Endongenous BDNF is required for long-term memory formation in the rat parietal córtex. Learn Mem 12(5),504-10, 2005.

18. Alonso M, Medina JH, Pozzo-Miller L. BDNF increases dendritic spine density in hippocampal CA1 pyramidal neurons via ERK 1/2 activation. Learm Mem, 11:172-78, 2004

19. Kapczinski F, Frey BN, Andreazza AC, Kauer-Sant'Anna M, Cunha ABM, Post RM. Increased oxidative stress as a mechanism for decreased BDNF levels in acute manic episodes. Rev.Bras. Psiquiatr 30 (3):243-5, 2008.

20. Kamat, J. P., Devasagayam, T. P., Priyadarsini, K. I., Mohan, H., and Mittal, J. P. Oxidative damage induced by the fullerene C 60 on photosensitization in rat liver microsomes. Chemico-Biological Interactions 1998; 114: 145 - 159.

21. Sayes, C., M., Gobin, A., M., Ausman, K.,D.,Mendez, J., West, J.,L., Colvin, V.,L. Nano-C60 cytotoxicity is due to lipid peroxidation. Biomaterials 2005; 26: 7587-7595

22. Packer L., Tritschler H.J., Wessel K. Neuroprotection by the metabolic antioxidant □-lipoic acid. Free Radical Biology and Medicine 1997; 22: 359-378.

23. Cardozo-Pelaez F, Brooks PJ, Stedeford T., Song S., Sanchez-Ramos J. DNA damage, repair, and antioxidant systems in the brain region: a correlative study. Free Radical Biology and Medicine 28: 779-785, 2000.

24. Qiao D., Seidler F.J., Slotkin T.A. Oxidative mechanisms contributing to the developmental neurotoxicity of nicotine and chlorpyrifos. Toxicol. Appl. Pharmacol., 206: 17-26, 2005

25. Balu M., Sangeetha P., Haripriya D., Panneerselvam C. Rejuvenation of the antioxidant system in the central nervous system of aged rats by grape seed extract. Neuroscience Letters 383: 295-300, 2005.

26. Xiao, L., Takada, H., Maeda, K., Haramoto, M., and Miwa, N. Antioxidant effects of water-soluble fullerene derivatives against ultraviolet ray or peroxylipid through their action of scavenging the reactive oxygen species in human skin keratinocytes. Biomed. Pharmacother 2005; 59, 351-358.

Normas da Revista

Guide for Authors
The peer review submission system for *Biomaterials* is located here: http://ees.elsevier.com/biomat/
The Elsevier Editorial System (EES) is a web-based submission and review system. Authors may submit manuscripts and track their progress through the system to publication. Reviewers can download manuscripts and submit their opinions to the editor. Editors can manage the whole submission/review/revise/publish process.

Please register at: http://ees.elsevier.com/biomat/

Referees, whose names are not normally disclosed to the authors, will study all contributions which the Editor-in-Chief deems to be of sufficient significance and interest to be sent for peer review. The criteria by which this initial assessment is made include relevance to the scope of the journal, the originality of the work and its significance to the broad development of the field of biomaterials.

Before You Begin
All authors, especially those submitting to the journal for the first time, are encouraged to read the following document authored by the Editor in Chief, which gives an overview of the journal as well as the writing and selection process of academic publishing as it relates to *Biomaterials*: Writing Papers for *Biomaterials* .

Ethics in Publishing: For information on Ethics in Publishing and Ethical guidelines for journal publication see http://www.elsevier.com/authorethics and http://www.elsevier.com/ethicalguidelines.

Mandatory Author Declaration: In addition to uploading manuscripts and figures, it is required that the corresponding author of each manuscript uploads a separate Author Declaration. All authors must sign this declaration; the corresponding author may not sign on behalf of other authors.
This Declaration covers a number of logistic and ethical issues. Please use the template given above for this Declaration. Authors may save this template, obtain the required signatures and then upload it as an integral part of their submission. The editorial process will not begin until and unless all authors have signed the declaration.

N.B.: The Author Declarationis independent of, and in addition to, the "Journal Publishing Agreement" agreement which is issued on acceptance.

Amendments to Manuscripts
In order to maintain the integrity of the scientific record, the version that is published in an issue on ScienceDirect and in print MUST be identical. One set of page proofs will be sent to the corresponding author. Please note that authors are urged to check their proofs carefully before return, but corrections are restricted to typesetting errors only. Proofs are NOT to be considered as drafts. No changes in, or additions to, the accepted (and subsequently edited) manuscript will be allowed at this stage. Proofreading is solely the responsibility of the corresponding author.

Copyright
All authors must sign the "Journal Publishing Agreement" before the article can be published. An e-mail (or letter) will be sent to the corresponding author confirming receipt of the manuscript together with an agreement form or a link to the online version of this agreement. This transfer agreement enables Elsevier Ltd to protect the copyrighted material for the authors, but does not relinquish the author's proprietary rights. As an author you (or your employer or institution) retain certain rights; for details you are referred to: http://www.elsevier.com/authorsrights.

The copyright transfer covers the exclusive rights to reproduce and distribute the article. Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations (please consult http://www.elsevier.com/permissions). If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases: please consult http://www.elsevier.com/permissions.

Role of the Funding Source
You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the

writing of the report; and in the decision to submit the paper for publication. If the funding source(s) had no such involvement then this should be stated. Please see http://www.elsevier.com/funding.

Funding Bodies Agreements and Policies
Elsevier has established agreements and developed policies to allow authors whose articles appear in journals published by Elsevier, to comply with potential manuscript archiving requirements as specified as conditions of their grant awards. To learn more about existing agreements and policies please visit http://www.elsevier.com/fundingbodies.

Manuscript Preparation

General: Manuscripts must be word processed (preferably in Word format), double-spaced with wide margins and a font size of 12 or 10 pt. Files prepared in LaTEX are not supported. Figure captions should be in Arial font 9pt. Please check the current style of the journal, particularly the reference style (Vancouver), and avoid excessive layout styling as most formatting codes will be removed or replaced during the processing of your article. In addition, do not use options such as automatic word breaking, justified layout, double columns or automatic paragraph numbering (especially for numbered references). However do use bold face, italic, subscripts, superscripts etc. The corresponding author should be identified (include a Fax number and E-mail address). Full postal addresses must be given for all co-authors. The Editors reserve the right to adjust style to certain standards of uniformity. The preferred style is: Surname, Initials, Department, Institution, City/State, Postal Code, Country. Authors should retain copies of all versions of their manuscript submitted to the journal. Authors are especially requested to be vigilant over the submission of the correct version of the manuscript at the various stages of the editorial process.

English Language: Manuscripts should be proof-read and have English language errors corrected before submission as we may have to return papers due to poor language usage. Authors who require information about language editing and copyediting services pre- and post-submission please visit http://www.elsevier.com/languagepolishing or contact authorsupport@elsevier.com for more information.

Paper Length: Authors are urged to write as concisely as possible.

Abstracts: All manuscripts are to be supplied with an Abstract of about 100-200 words in length.

Keywords: Authors must provide 4-6 keywords for indexing purposes. A keyword list can be found at the end of these instructions.

Text: Follow this order when typing manuscripts:Title, Authors, Affiliations, Abstract, Keywords, Introduction, Materials and Methods, Results, Discussion, Conclusions, Acknowledgements, References, Appendix (where necessary), Figures, Figure Captions and Tables, Supplementary Data. The corresponding author should be identified with an asterisk and footnote. All other footnotes (except for table footnotes) should be identified with superscript Arabic numbers. An abbreviated title for use as a running headline should also be supplied. Authors are requested to acknowledge funding sources for the work.

Units: The SI system should be used for all scientific and laboratory data; if, in certain instances, it is necessary to quote other units, these should be added in parentheses. Temperatures should be given in degrees Celsius. The unit 'billion' (109 in America, 1012 in Europe) is ambiguous and must not be used. If a large number of symbols are used it is helpful if authors submit a list of these symbols and their meanings.

References: All publications cited in the text should be presented in a list of references following the text of the manuscript. In the text refer to references by a number in square brackets on the line (e.g. Since Peterson[1]), and the full reference should be formatted according to the permutation of the Vancouver reference system with numbers in the text. It is suggested that authors refer to the following link for a comprehensive overview of the Vancouver reference styles: Vancouver Reference Styles

Examples of formatting follow:

1. Driessens FCM, Boltong MG, Bermudez O, Planell JA. Formulation and setting times of some calcium orthophosphate cements: a pilot study. J Mater Sci: Mater Med 1993;4:503-508.
2. Nancollas H. In vitro studies of calcium phosphate crystallisation. In: Mann S, Webb J, Williams RJP, editors. Biomineralization. Chemical and biochemical perspectives. New York: VCH, 1989. p. 157-182.
3. Brown W, Chow LC. Combinations of sparingly soluble calcium phosphates in slurries and paste as mineralizers and cements. US Patent No. 4612053, 1986.

N.B.: ?Et al? must be used after the first 6 authors have been named. *Biomaterials* does not use the publication month or day.

Online Sources: References to online sources should contain at a minimum the full URL and year the source was accessed. Furthermore, if known, the following information should be given: author names, dates, reference to a source publication. Web

references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

Examples of formatting follow:

1. UK House of Commons Science and Technology Committee. Scientific Publications: Free for All? Tenth Report of Session 2003-4 Volume 1. London: The Stationary Office Ltd. Online. 2004 July. Available from URL: http://www.publications.parliament.uk/pa/cm200304/cmselect/cmsctech/399/39902.html
2. Wellcome Trust. Economic Analysis of Scientific Research Publishing. Histon, UK: Wellcome Trust. Online. 2003. Available from URL: http://www.wellcome.ac.uk/doc_wtd003181.html

Artwork

Electronic artwork
General points
• Make sure you use uniform lettering and sizing of your original artwork.
• Save text in illustrations as "graphics" or enclose the font.
• Only use the following fonts in your illustrations: Arial, Courier, Times, Symbol.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Provide captions to illustrations separately.
• Produce images near to the desired size of the printed version.
• Submit each figure as a separate file.

A detailed guide on electronic artwork is available on our website: http://www.elsevier.com/artworkinstructions
You are urged to visit this site; some excerpts from the detailed information are given here.
Formats
Regardless of the application used, when your electronic artwork is finalised, please "save as" or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
EPS: Vector drawings. Embed the font or save the text as "graphics".
TIFF: color or grayscale photographs (halftones): always use a minimum of 300 dpi.
TIFF: Bitmapped line drawings: use a minimum of 1000 dpi.
TIFF: Combinations bitmapped line/half-tone (color or grayscale): a minimum of 500 dpi is required.
DOC, XLS or PPT: If your electronic artwork is created in any of these Microsoft Office applications please supply "as is".
Please do not:
• Supply embedded graphics in your wordprocessor (spreadsheet, presentation) document;
• Supply files that are optimised for screen use (like GIF, BMP, PICT, WPG); the resolution is too low;
• Supply files that are too low in resolution;
• Submit graphics that are disproportionately large for the content.

Color artwork
Please make sure that artwork files are in an acceptable format (TIFF, EPS or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color on the Web (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article. Please indicate your preference for color in print or on the Web only. For further information on the preparation of electronic artwork, please see http://www.elsevier.com/artworkinstructions. Please note: Because of technical complications which can arise by converting color figures to "gray scale" (for the printed version should you not opt for color in print) please submit in addition usable black and white versions of all the color illustrations.

Video Data
Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly relate to the video file's content. In order to ensure that your video or animation material is directly usable, please provide the files in one of our recommended file formats with a maximum size of 30 MB and running time of 5 minutes. Video and animation files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages at http://www.elsevier.com/artworkinstructions. Note: since video and animation cannot be embedded in the print version of the journal, please provide text for both the electronic and the print

version for the portions of the article that refer to this content. Files can be stored on diskette, ZIP-disk or CD (either MS-DOS or Macintosh).

After Acceptance

Proofs: One set of page proofs in PDF format will be sent by e-mail to the corresponding author and should be returned within 48 hours of receipt. The average amount of time between acceptance and receipt of typeset proof is 6 working days. Papers are published in print within another 8 weeks upon receipt of author corrections. Corrections should be restricted to typesetting errors. Any queries should be answered in full. Please note that authors are urged to check their proofs carefully before return.

Elsevier now sends PDF proofs which can be annotated; for this you will need to download Adobe Reader© version 7 (or higher) available free from http://www.adobe.com/products/acrobat/readstep2.html. Instructions on how to annotate PDF files will accompany the proofs. The exact system requirements are given at the Adobe site: http://www.adobe.com/products/acrobat/acrrsystemreqs.html#70win. If you do not wish to use the PDF annotations function, you may list the corrections (including replies to the Query Form) and return to Elsevier in an e-mail. Please list your corrections quoting line number. If, for any reason, this is not possible, then mark the corrections and any other comments (including replies to the Query Form) on a printout of your proof and return by fax, or scan the pages and e-mail, or by post. Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes to the article as accepted for publication will only be considered at this stage with permission from the Editor. We will do everything possible to get your article published quickly and accurately. Therefore, it is important to ensure that all of your corrections are sent back to us in one communication: please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility. Note that Elsevier may proceed with the publication of your article if no response is received.

Track a Paper: Authors can track their paper status online after the paper has been accepted and forwarded to the Publisher. Enter your Elsevier reference number (JBMT xxx) and the Corresponding author's family name at the following web page: http://www.elsevier.com/trackarticle. Corresponding authors will receive an acknowledgement email from Elsevier with the reference number and the family name on it. Authors can also go to the 'track a paper' page by clicking onto the 'track a paper' button on the left hand side of the journal home page. .

Offprints: The corresponding author will be provided with a PDF of the article via email. The PDF is a watermarked version of the published article and includes a cover sheet with the journal cover image and a disclaimer outlining the terms of use. Additional paper offprints can be ordered by the authors. An order form with prices will be sent to the corresponding author.

Author Enquiries: For enquiries relating to the submission of articles (including electronic submission where available) please visit this journal?s homepage. You can track accepted articles at http://www.elsevier.com/trackarticle and set up e-mail alerts to inform you of when an article?s status has changed. Also accessible from here is information on copyright, frequently asked questions and more. Contact details for questions arising after acceptance of an article, especially those relating to proofs, will be provided by the publisher.