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FISIOLOGIA ANIMAL COMPARADA

AVALIAÇÃO DA CO-EXPOSIÇÃO *IN VITRO* DO ARSÊNIO E DO NANOMATERIAL ORGÂNICO FULERENO C_{60} EM HEPATÓCITOS DE ZEBRAFISH *Danio rerio*

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Sumário

RESUMO

As nanopartículas são materiais que possuem pelo menos uma dimensão na faixa de 1 a 100 nm e devido a sua capacidade de interagir com outras moléculas, os nanomateriais apresentam muitas aplicações biotecnológicas. Mesmo com a crescente demanda e utilização de produtos oriundos do campo da nanotecnologia, pouco se sabe dos impactos causados por estes aos sistemas biológicos. Dentre as nanopartículas podemos citar o fulereno (C_{60}), um composto orgânico lipofílico que, potencialmente, poderia servir como carreador de moléculas tóxicas. Tendo-se em vista o conceito de "cavalo de Tróia", onde contaminantes podem ter sua entrada em órgãos específicos potencializada por sua associação com nanopartículas, torna-se imprescindível conhecer as possíveis consequências biológicas desta associação. Hepatócitos de zebra fish, foram expostos ao arsênio, ao fulereno e em forma conjunta (As + C_{60}) por 4 h, período em que a viabilidade celular não foi alterada. A produção de espécies reativas de oxigênio foi reduzida ($p<0,05$) no grupo controle e na concentração 100µM As co-expostas com C_{60}; a concentração intracelular de GSH foi reduzida ($p < 0.05$) na concentração 2,5 µM As co-exposta com C_{60}, sendo relacionado com a ausência de dano lipídico (TBARS) na mesma concentração. Não houve diferenças ($p>0,05$) no nível de proteínas oxidadas. A capacidade antioxidante total foi significativamente maior nas amostras de As co-expostas com C_{60}, enquanto que a atividade da GST Ω foi reduzida no tratamento As (100µM) + $C_{60.}$ Todos os resultados encontrados neste trabalho são fortemente sustentados pela comprovação do papel de carreadores das nanopartículas, pois a concentração intracelular de arsênio nas células co-expostas com fulereno apresentou um resultado significativamente maior. Portanto conclui-se que nestas concentrações, durante o tempo de exposição e com o modelo experimental utilizado o nanomaterial fulereno co-exposto com o arsênio apresenta capacidade antioxidante. Futuros estudos são necessários para avaliar a biodisponibilidade intracelular de arsênio depois de ser ligado ao nanomaterial fulereno.

Palavras chave: Fulereno, arsênio, antioxidante, cavalo de Tróia, hepatócitos, peixe-zebra.

ABSTRACT

Nanoparticles are materials that have at least one dimension in the range of 1-100 nm and due to its ability to interact with other molecules, they have many biotechnological applications. Even with the increasing demand and use of products from nanotechnology, little is known about the impacts in biological systems. Among nanomaterials, fullerene (C_{60}) is a lipophilic compound that can enter the different cellular compartments and to serve as a carrier to other molecules. The semi-metal arsenic found in water, soil and air is -among several toxic molecules- one that can be considered interesting to evaluate their interaction with fullerene. Taking into account the concept of "Trojan Horse", where contaminants may have its entry into specific organs potentiated by its association with nanoparticles, becomes essential to understand the potential biological consequences of this association. Hepatocytes of zebrafish *Danio rerio* were exposed to arsenic, fullerene and As+C_{60} for 4 h, during which the cell viability was stable. Reactive oxygen species production was reduced in the control group and in the As 100 μM + C_{60} treatment; the intracellular concentration of glutathione (GSH) was reduced ($p< 0,05$) in the As 2.5 μM+C_{60} treatment, a result which is in agreement with the absence of lipid damage (TBARS) in the same group. There were no significant differences in oxidized proteins in any of treatment and control group. Total antioxidant capacity was significantly higher in both As concentrations co-exposed to C_{60}. The GSTΩ was reduced in the As 100 μM+C_{60} treatment. Cells co-exposed to C_{60} had a significantly higher accumulation of As, showing a "Trojan Horse" effect that, however, not resulted in higher cell toxicity. Instead, co-exposure with C_{60} showed to reduce As cellular injury. We concluded that in the tested conditions and with this experimental model, the fullerene presented an antioxidant pattern. Further studies are needed to access the bioavailability of arsenic in the cells after being linked to the fullerene nanomaterials.

Key words: Fullerene, arsenic, antioxidant, Trojan horse, hepatocytes, zebrafish.

INTRODUÇÃO

As nanopartículas são materiais que possuem pelo menos uma dimensão na faixa de 1 a 100 nm. Essa característica faz com que suas propriedades físicas e químicas sejam distintas daquelas verificadas em materiais de maior tamanho (Oberdörster, 2004a). Devido à grande relação superfície/volume em função da sua escala nanométrica, a capacidade de interagir com outras moléculas, incluindo biomoléculas, torna-se muito maior e é esta característica que brinda aos nanomateriais muitas aplicações biotecnológicas (Oberdörster *et al*., 2005; Bhatt *et al*., 2010).

Apesar da crescente demanda e utilização de produtos oriundos do campo da nanotecnologia, pouco se sabe dos impactos causados por estes nanocompostos aos sistemas biológicos. Em virtude dessa carência de informações vem se desenvolvendo um ramo da ciência denominada *nanotoxicologia*, que objetiva avaliar os efeitos dos nanomateriais nos organismos vivos (Oberdörster *et al.*, 2005; Oberdörster *et al.*, 2007;Kahru *et al*.,2010).

Dentre os nanomateriais podemos citar o fulereno (C_{60}) Fig.1, descoberto em 1985, um nanomaterial que possui 60 átomos de carbono ligados como uma gaiola com uma estrutura semelhante a uma bola de futebol composta por 12 pentágonose 20 hexágonos. Este compostotornou-se popular entre os químicos, tanto pela sua beleza estrutural quanto pela sua versatilidade para a síntese de novos compostos químicos (Kroto,2006).

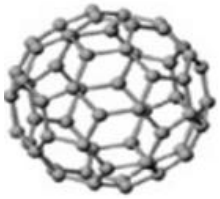

Figura 1: Fulereno (C_{60}).

Devido à sua forma tridimensional, suas ligações insaturadas e sua estrutura eletrônica, os fulerenos apresentam propriedades físicas e químicas únicas que podem ser exploradas em várias áreas da bioquímica e da medicina (Shinohara *et al*., 2009). Assim, o fulereno (ou o fulereno funcionalizado) é utilizado para diversos fins, como por exemplo, a composição de cosméticos, lubrificantes, complementos alimentares, materiais de construção, vestuário, materiais para contenção de derramamento de petróleo, isoladores acústicos e térmicos, materiais semicondutores, catalisadores químicos e carreadores de fármacos (Loutfy *et al*., 2002; Kahru *et al*.,2010).

O fulereno, por ser um composto lipofílico, pode atravessar membranas celularese localizar-se preferencialmente nas mitocôndrias, citoplasma, lisossomos, nos núcleos celulares, e no organismo pode também atravessar a barreira hematoencefálica (Porter *et al*., 2007). Em função desta capacidade de ingressar a diferentes compartimentos celulares, é importante se perguntar a respeito da toxicidade exercida por este composto.

A presença de fulereno no ambiente pode alterar a capacidade de detoxificação de espécies ativas de oxigênio (EAO) dos organismos, levando em alguns casos, a uma situação de estresse oxidativo (Oberdörster, 2004; Sayes *et al.,* 2005; Shinohara *et al*.,2009).

Autores como Zhu *et al.* (2008), estudaram as respostas em espécies juvenis do peixe dourado *Carassius auratus*, expostos a concentrações sub-letais de fulereno (0,04 mg/L - 1,0 mg/L), e observaram o aumento da atividade de enzimas antioxidantes como a superóxido dismutase (SOD) e a catalase (CAT) no fígado destes peixes. No mesmo estudo, um dos principais antioxidantes intracelulares, a glutationa reduzida (GSH), diminuiu em todos os órgãos analisados. Além disso, o peso corporal e o comprimento total dos peixes diminuíram significativamente indicando que o C_{60} teve um efeito

inibitório sobre o crescimento. Os autores observaram também um aumento da peroxidação lipídica (LPO) no fígado após a exposição a 1mg/L de fulereno, enquanto nas brânquias e no cérebro houve uma redução da LPO.

Em um trabalho prévio de Oberdörster (2004), a queda na concentração de GSH também foi observado em brânquias do peixe *Micropterus salmoides* expostos ao fulereno, tendo sido também observada peroxidação lipídica no cérebro dos peixes após 48h de exposição a 0,5 mg de C_{60}/L, sugerindo que neste tempo e nesta concentração o fulereno pode induzir uma situação de estresse oxidativo.

Além dos estudos citados acima, outros trabalhos descrevem o fulereno como um produtor de radicais livres com um forte potencial oxidativo através da foto-ativação (faixa visível e UVA), induzindo toxicidade em culturas celulares (Wang e Joseph, 1999; Mori *et al*., 2006,Shinohara *et al*.,2009).O estudo de Kamat *et al*. (1998), analisou o efeito do fulereno foto ativado (UV luz visível) em fígado de ratos, o que induziu um significante dano lipídico e protéico.

Entretanto, outros estudos sugerem que o C_{60} funciona como um poderoso antioxidante. Como exemplo pode ser citado o trabalho de Usenko *et al.* (2007), onde foi observado que embriões de peixe zebra expostos ao fulereno mostraram um menor índice de estresse oxidativo que pode ser evidenciado pela diminuição nos níveis de peroxidação lipídica no cérebro, demonstrando que o fulereno poderia estar agindo como um antioxidante. O trabalho de Zhu *et al.* (2008) também mostrou em embriões de peixe zebra que os níveis de peroxidação lipídica diminuíram após exposição ao fulereno, especialmente nas brânquias e no cérebro. Ainda, o trabalho de Gharbi *et al.* (2005) verificou em ratos, que o fulereno agiu como um poderoso antioxidante *in vivo*, sem apresentar toxicidade aguda ou subaguda. Bickley e Mcclellan-Green (2008)

também observaram uma modulação no sistema antioxidante evidenciado pelo aumento nos níveis de GSH no peixe *Fundulus heteroclitus*.

Estas divergências de informações fazem com que o estudo de avaliação dos possíveis efeitos deletérios provocados por nanomateriais oriundos da nanotecnologia (nanotoxicologia) aumentasse nos últimos anos (Oberdörster *et al*., 2005; Oberdörster *et al*., 2007; Kahru *et al*., 2010) justamente na procura de se estabelecer em que condições ou situações estas partículas podem se comportar como anti ou pró-oxidantes e, neste último caso, qual seu potencial efeito tóxico.

Além disso, a possibilidade da associação do fulereno com outros contaminantes torna o estudo de sua possível toxicidade ainda mais importante, como demonstrado por Limbach *et al*. (2007), onde foi observado que a produção de EAO nas células de epitélio pulmonar expostas ao cobalto e ao manganês co-expostas à nanopartículas de sílica foi até oito vezes maior em relação aos seus controles. Estes autores sugerem que as nanopartículas de sílica podem servir como carreadores de metais, levando-os a compartimentos celulares que não seriam acessíveis caso não houvesse a presença do nanomaterial. Os nanomateriais estariam agindo, desta forma, como um "cavalo de tróia" para outras moléculas tóxicas (Limbach *et al.*, 2007).No trabalho de Sun *et al*. (2008), foi evidenciado o papel de carreadores das nanopartículas, onde a co-exposição de nanopartículas de dióxido de titânio com arsenito ocasionou, após 25 dias de exposição, um aumento de 44% na bioacumulação deste metalóide em brânquias, vísceras e músculo de carpa.

Outras nanopartículas conhecidas como as de dióxido de titânio mostraram induzir dano de DNA em celomócitos do poliqueto estuarino *Arenicola marina* expostos ao sedimento enriquecido com as nanopartículas (Galloway *et al.*, 2010). Nanopartículas de ouro e prata também parecem exercer efeitos em hepatócitos do peixe

Oncorhynchus mykiss. As nanopartículas de prata mostraram efeitos citotóxicos que foi evidenciada pela diminuição da integridade da membrana e atividade metabólica enquanto que nanopartículas de ouro mostraram aumentar de forma significativa a produção de espécies ativas de oxigênio (EAO) (Farkas *et al.*, 2010).

Estudos que contemplem a interação com compostos tão pouco estudados como os nanocompostos, são de extrema importância, uma vez que no ambiente é praticamente impossível a contaminação por uma única molécula tóxica. Tendo-se o arsênio entre as moléculas tóxicas que podem ser consideradas interessantes para avaliar sua interação com o fulereno.

O arsênio (As) é um semi-metal encontrado na água, no solo e no ar, sendo um dos responsáveis pela contaminação dos sedimentos (Hamilton, 2000; Sharma *et al*, 2009). O As é proveniente de fontes naturais e antrópicas, como efluentes de metais não-ferrosos, efluentes e emissões de queima de carvão, bem como em sedimentos próximos a indústrias de fertilizantes por coexistir com grupos fosfato (Popovic *etal*., 2001, Mirlean e Rosenberg, 2006). Na região sul do país (RS), a cidade de Rio Grande vem sofrendo com a problemática de contaminação por As no sedimento e também na água intersticial (Mirlean *et al*., 2003; Mirlean e Roisenberg, 2006). Esta contaminação está principalmente vinculada as indústrias de fertilizantes, não pelo uso direto do As no produto, mas pelo fato que este metalóide é um contaminante natural de grupos fosfato.

Devido à sua presença no ambiente, a probabilidade de exposição ao As é elevada, colocando em risco a saúde dos organismos que nele vivem. Este elemento é encontrado em várias formas químicas de oxidação e provoca efeitos adversos para a saúde, incluindo câncer de pele, pulmão, fígado, bexiga e outros tecidos (Hughes, 2002;Sharma*et al.*, 2009). Em alguns animais aquáticos existem evidências de que o arsenito de sódio: (a) aumenta a citotoxicidade em linhagens celulares (Seok *et al.*

2007); (b) causa diminuição na atividade de enzimas antioxidantes no poliqueto *Laeonereis acuta* e no peixe *Clarias batrachus (*Bhattacharya and Bhattacharya, 2007); (c) aumenta a resposta antioxidante em brânquias do peixe zebra exposto por dois dias (Ventura-Lima *et al*. 2009a); (d) causa estresse oxidativo no fígado do peixe dourado *Carassius auratus (*Bagnyukova et al., 2007)*;* (e) induz alterações no sistema antioxidante em diferentes tecidos em carpa *Cyprinus carpio* (Ventura-Lima *et al*. 2009b).

No estudo de Xu *et al.* (2010) foi investigado em células hepáticas humanas os efeitos tóxicos do arsenito de sódio ($NaAsO_2$) na concentração de 20 µM após 24h de exposição. Os resultados mostraram que com este tempo e nesta concentração o arsenito induz apoptose, aumenta significativamente a produção de espécies ativas de oxigênio e também a peroxidação lipídica.

O metabolismo do As tem um papel importante na sua toxicidade, na água ele apresenta-se sobre tudo sob condições oxidantes na forma inorgânica pentavalente (As^{V}), mas quando ingerido por animais ou seres humanos passa por transformações bioquímicas que o reduzem a arsenito (As^{III}), que é cerca de 60 vezes mais tóxico que o arsenato (Arkter *et al*., 2005).

Alguns autores sugerem que a glutationa está envolvida no processo de biotransformação do arsenato, o que tem sido mostrado na formação do complexo arsênio triglutationa ($As(GS_3)$)formado com resíduos de cisteína e três moléculas de GSH, em mamíferos, subsequentemente o As^{III} inorgânico é enzimaticamente metilado através da ação da arsenito metil transferase (Kobayashi *et al.*, 2005). A glutationa-S-transferase ômega (GST-Ω) também está envolvida no processo de biotransformação do arsênio inorgânico e nos processos de metilação do arsênio. Essas enzimas da família da GST são multifuncionais estando envolvidas no processo de detoxificação de

xenobióticos, sendo encontradas em quase todos os organismos vivos (Sakurai *et al.*, 2005; Ventura-Lima *et al.*, 2010).

O arsenito é a forma mais tóxica do arsênio e por ser mais instável é mais reativo, reagindo com moléculas biológicas. Por possuir grande afinidade por grupos sulfidrilas de proteínas (Wang *et al.*, 2004), o arsenito pode levar a inativação de muitas enzimas e receptores celulares (Tseng, 2004). Ramanathan *et al.* (2003) relatam que o arsenito é um inibidor da piruvato desidrogenase e da α-cetoglutarato desidrogenase, o que afeta o ciclo de Krebs, que poderá culminar com uma diminuição na síntese de ATP, gerando alterações em todo o organismo inclusive na síntese de GSH, podendo levar a um dano celular progressivo (Mandal e Suzuki, 2002).

Todas as formas de As inorgânico, mesmo em baixas concentrações e com pouco tempo de exposição, podem desencadear a formação excessiva de EAO (Laparra *et al.*, 2008). Para proteger as células contra os possíveis danos oxidativos provocados pelas EAO, os organismos aeróbios utilizam defesas antioxidantes, incluindo as enzimáticas, como a catalase (CAT), glutationa redutase (GR), glutationa-S-transferase ômega (GSTΩ) e algumas não enzimáticas, como a glutationa (GSH) e o ácido lipóico (Shila *et al.*, 2005).

A super família da glutationa-S-transferase (GST) é dividida em diferentes classes: alfa, mu, pi, teta, zeta, sigma e ômega (GST Ω) (Ventura-Lima *et al.*, 2010). A GST Ω desempenha papeis diferentes das outras GST, tendo sido descrito duas isoformas. A primeira (GST Ω1) está envolvida nos processos de redução do arsenato (As^{v}), do acido monometilarsenato (MMA^{v}) e do acido dimetilarsenato (DMA^{v}). A segunda (GST Ω2) recentemente identificada, também pode catalisar a redução do acido monometil arsenato (MMA^{v}) e do acido dimetilarsenato (DMA^{v}), mas possui uma baixa atividade redutase para o DMA^{v} (Agusa *et al.*, 2010).

A glutationa (GSH) é o mais abundante agente redutor presente nas células. Este tripeptídeo é sintetizado pela atividade combinada das enzimas, a glutamato cisteína ligase (GCL) e a glutationa sintetase (White *et al.*, 2003). A glutationa forma um sistema que mantém o ambiente intracelular reduzido e atua como uma defesa antioxidante primária contra a geração excessiva de EAO (Ochi, 1997). O sistema GSH tem um papel de extrema importância na proteção celular contra exposição ao arsênio inorgânico, como demonstrado por Ventura-Lima *et al.* (2009a), onde foi observado que em brânquias do peixe zebra expostos ao arsênio houve um aumento nos níveis de GSH paralelo a um aumento na atividade da GCL, sendo que não foi observado dano oxidativo em nível lipídico.

No entanto pouco se sabe da interação de nanomateriais com moléculas tóxicas de importância ambiental. Estas interações poderiam gerar várias e sérias consequências toxicológicas, incluindo a geração de espécies ativas de oxigênio com a consequente indução de dano oxidativo. Com a crescente demanda de produtos oriundos da nanotecnologia, torna-se imprescindível se conhecer as possíveis consequências biológicas da associação do fulereno com importantes contaminantes ambientais, como o As, ainda mais em um momento em que o uso dos produtos oriundos dessa nova tecnologia cresce a cada dia.

Portanto, sabendo-se que contaminantes podem ter sua entrada em órgãos específicos potencializada por sua associação com o fulereno, e considerando-se os divergentes resultados em relação aos efeitos deste nanocomposto, torna-se relevante o estudo da relação do fulereno com o semimetal arsênio o qual é encontrado na água, no solo e no ar, sendo um dos responsáveis pela contaminação dos sedimentos.

OBJETIVOS

O presente estudo teve como objetivo avaliar os efeitos da co-exposição de arsênio e fulereno em uma linhagem celular de hepatócitos do peixe zebra *Danio rerio*, considerando a concentração de espécies ativas de oxigênio (EAO), concentração do antioxidante glutationa (GSH), dano oxidativo lipídico, dano oxidativo protéico, capacidade antioxidante total, atividade da GST ômega e incorporação de arsênio nas células utilizadas como modelo biológico.

ARTIGO

Revista: Toxicology in Vitro
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In vitro evaluation of co-exposure of arsenium and an organic nanomaterial (fullerene, C_{60}) in zebrafish hepatocytes

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Abstract

Taking into account the concept of "Trojan Horse", where contaminants may have its entry into specific organs potentiated by its association with nanoparticles, the aim of this study was to analyze the join toxic effects induced by an organic nanoparticle, fullerene (C_{60}) with the metalloid arsenic (As). Hepatocytes of zebrafish *Danio rerio* were exposed to As, C_{60} or As+C_{60} for 4 h, not altering cells viability. Intracellular reactive oxygen species concentration was reduced in cells exposed only to the C_{60} (1 mg/L) and in the treatment of As 100 μM+C_{60}. Co-exposure with C60 abolished the peak of the antioxidant glutathione (GSH) registered in cells exposed to the lowest As concentration (2.5 μM). A similar result was observed in terms of lipid damage (TBARS). Total antioxidant capacity was significantly higher in both As concentrations co-exposed to C_{60} when compared with the control group. Activity of glutathione-S-transferase omega (GST Ω), a limiting enzyme in the methylation pathway of As, was reduced in the As 100 μM+C_{60} treatment. Cells co-exposed to C_{60} had a significantly higher accumulation of As, showing a "Trojan Horse" effect that, however, not resulted in higher cell toxicity. Instead, co-exposure with C_{60+} As showed to reduce cellular injury.

Key words: Fullerene, arsenic, antioxidant, glutathione-S-transferase omega, hepatocytes, zebrafish.

1. Introduction

Despite the growing demand for products originated from nanotechnology, little is known about the impacts to biological systems (Kahru and Dubourguier, 2010).Among nanoparticles, fullerene (C_{60} and C_{70}, among others), is a carbon molecule possessing only carbon atoms, which a chemical structure resembling a soccer ball. Due to its three- dimensional shape, its unsaturated bonds and its electronic structure, fullerenes have unique chemical and physical properties which that can be exploited in several areas of biochemistry and medicine (Usenko *et al*., 2008). Because of its lipophilic nature, it can cross membranes and the blood-brain barrier (Porter *et al*., 2007). Taking into account its potential ability to move to different cellular compartments, concerns about the toxicity exerted by this nanoparticle has been raised (Oberdörster, 2004; Oberdörster *et al.,* 2007; Kahru and Dubourguier, 2010).

It has been registered in fish species after exposure to fullerene, increased activity of antioxidant enzyme such a catalase (CAT) in liver, whereas the antioxidant glutathione (GSH) decreased in all tissues tested. Moreover, the total animal weight and length significantly decreased showing that fullerene had an inhibitory effect on fish growth (Zhu *et al*., 2008). In previous work, it was found a decrease of the antioxidant glutathione (GSH) levels in gills of fish *Micropterus salmoides* and also lipid peroxidation in the brain after 48h of exposure to 0.5mg of C_{60}/L, indicating that in this case, this nanomaterial presented potential to generate oxidative stress (Oberdörster, 2004). Other studies described fullerene as a free radical producer, with a strong oxidative potential by photo activation, inducing toxicity in cell culture (Wang *et al*., 1999; Kamat, 2002; Mori *et al*., 2006; Shinohara *et al*., 2009).

On the other hand, some studies have reported fullerene as being a powerful antioxidant. For example, it was observed that zebrafish embryos exposed to fullerene showed lower lipid peroxidation in brain (Usenko *et al*., 2007). Other study also employing zebrafish embryos showed that the levels of lipid peroxidation (LPO) decreased after exposure to fullerene, especially in the gills and brain (Zhu *et al*., 2008). Exposure of fullerene in rats also showed *in vivo* antioxidant effects, without presenting acute or sub-acute toxicity (Gharbi *et al*., 2005). This divergence of information led to assessment studies on the deleterious effects of carbon nanoparticles, such as fullerene, to be increased in recent years (Oberdörster *et al*., 2005; Aschberger *et al*., 2010), raising the need of establish under which conditions these nanoparticles can behave as anti- or pro-oxidants.

Limbach *et al.* (2007) observed that the production of reactive oxygen species (ROS) in lung epithelial cells exposed to cobalt and manganese co-exposed to silica nanoparticles was 8 times higher compared to the controls. The authors proposed that silica nanoparticles acted as carries of metals, playing a ´Trojan horse´ role. Also, other study reported that the co-exposure to nanoparticles of titanium dioxide with arsenite caused a 44% increase of bioaccumulation of arsenite in gills, viscera and muscle of carp after 25 days of exposure (Sun *et al*., 2009).

Arsenic is a toxic molecule interesting to evaluate its potential interaction with fullerene. Is a metalloid found in water, soil, and air from natural and anthropogenic sources, existing both in inorganic and organic forms, with different oxidation states (Ventura-Lima *et al.*, 2011). The metabolism of arsenic has an important role in its toxic effects. Inorganic arsenic is metabolized by a sequential process involving a two-

electron reduction of pentavalent arsenic to trivalent arsenic, followed by oxidative methylation to pentavalent organic form (Thomas *et al.,* 2001). The reduction can occur non-enzymatically in the presence of a thiol such as glutathione (GSH) (Delnomdedieu *et al*., 1994; Xuexia *et al*., 2008). All forms of inorganic arsenic, even at low concentration and short exposure time can trigger ROS generation (Schuliga *et al.,* 2002; Chowdhury *et al.,* 2010; Ventura-Lima *et al.,* 2011).

Enzymes of the glutathione-S-transferase (GST) type are a multifunctional group of proteins involved in the detoxification of xenobiotics, being found in almost all living organisms. In this group it is included the omega glutathione-S-transferase omega (GST Ω), acting in the process of arsenic methylation (Sakurai *et al*., 2005; Ventura-Lima *et al.,* 2011). In this way, reductions of monomethylarsenate acid (MMA^V) to monomethylarsenite acid (MMA^{III}), and dimethylarsenate acid (DMA^V) to dimethylarsenite acid (DMA^{III}) are catalyzed by GSTΩ. This enzyme is crucial for the pathway of methylation of inorganic As, since only arsenic species that have an oxidation state of +3 can be methylated (Chowdhury *et al*., 2006).

Taking into account the ´Trojan horse´ effect (Limbach *et al*., 2007), it can be considered the possibility of fullerene increase As toxicity. Furthermore, with the rapid growth of nanotechnology, it becomes essential to understand the potential biological consequences of the association of fullerene with important contaminants, such as arsenic, especially at a time when the use of products derived from these new technologies is increasing every day (Fischer and Chanw, 2007).

2. Materials and methods

2.1. Preparation of fullerene suspensions

Previous evidences showed that preparation of fullerene stock solution with organic solvents enhanced its toxicity (Zhu et al., 2006; Henry et al., 2007). So, an aqueous fullerene suspension was prepared, where two hundred milligrams of fullerene (SES research, purity: 99%) were diluted in 1L MilliQ water and kept under constant stirring and fluorescent light during 2 months. In this way, any toxic effects that may be registered will not be due to presence of solvents that can be trapped in these aggregates, increasing fullerene toxicity (Oberdörster *et al*., 2006; Henry *et al*., 2007). After stirring, suspensions were centrifuged at 25000 x g for 1 hour at 15°C to promote sedimentation of large aggregates and sequentially filtered through 0.45 and 0.20 μm pore membranes. Fullerene concentration was estimated by measuring total carbon concentration with a TOC-V CPH (Shimadzu) total organic carbon analyzer. The final concentration of fullerene used in cell bioassays was 1mg/L. Filtered C_{60} suspensions were characterized using a JEOL JSM 1200 EX II transmission electron microscopy operating at 100 kV. Samples of about 30 μl of C_{60} suspension were disposed onto 300 mesh TEM grids (SPI) coated with Formvar. Analysis was performed after 24 h in order to allow sample evaporation (Lyon *et al.*, 2006).

2.2. Preparation of arsenic solutions

After previous tests performed by our research group using the zebrafish liver cell line, the nominal concentrations of As^{III} solution selected to the assay were 2.5 μM and 100 μM. Arsenite solution was prepared using $NaAsO_2$ (VETEC) dissolved in ultrapure water, always prepared on the same day of the beginning of the assay. A previous study

reported the use of final concentrations ranging from 0 to 300μM of arsenite during 24 h for the same cell line (Seok *et al*., 2007).

2.3. Cell culture

Zebrafish hepatocyte cultures (strain ZFL), obtained from American Type culture collection(ATCC) were kept at 28^{o}Cin RPMI- 1640 culture medium supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic. Cells (10^{6}⁄mL) were incubated in 24 well cell culture plates and let to adhere for 24 h, and then, cells were exposed to 2 arsenic concentrations (2.5 and 100 μM) and fullerene (1 mg/L) for 4 hours. Four replicates were employed for each treatment: Control (medium culture and ultrapure water); 2.5 μM of As^{III}; 100 μM of As^{III}; Control+C_{60} (only fullerene added); 2.5 μM of As^{III}+C_{60}; 100 μM of As^{III}+C_{60}. At least two assays were performed for each measurement (see below). After exposure in the dark, the cells were washed in PBS, re-suspended with trypsin (0.25 % v/v) and stored for later biochemical and analytical analysis.

2.4. Estimation of the cells number

Cell count was performed with 800 μL of cell suspension (four cells pool). The cells number of each pool was count under a light microscope at 40 X. It was made serial dilutions (100%, 75%, 50% and 25%) for each cells pool, with culture medium. After this, the absorbance of each cell suspension was determined in an ELISA plate reader (Biotek ELx 800) at 630 nm. With these dilutions it was constructed a standard curve, fitting the absorbance to the cells number measured by microscopy. Then, the cells number of each treatment was estimated by measuring its absorbance at 630 nm.

2.5. Determination of mitochondrial dehydrogenases functionality

Mitochondrial dehydrogenases functionality was determined by MTT assay (2-(4, 5-dimethyl-2-thiazolyl)-3, 5-diphenyl-2H-tetrazolium bromide, after 30 minutes at 28°C in the dark, the plate was centrifuged at 1100rpm for 7 min, the supernatant discarded, DMSO added and read with an ELISA plate reader (Biotek Lx 800) at 490 nm.

2.6. Determination of the intracellular reactive oxygen species concentration (ROS)

Immediately after exposure, cells were centrifuged at 1.100 rpm for 5 min at 10^{o}C. The cell pellet was re-suspended in PBS containing 2,7´diclorodihidrofluorescein diacetate (H_2DCF- DA; 40 μM). Fluorescence was read at 28^{o}C during 70 min, using a plate reader fluorimeter (Victor 2, Perkin Elmer), with excitation and emission wavelength of 485 and 520 nm, respectively. ROS levels were expressed in terms of fluorescence area, after fitting fluorescence data to a second order polynomial and integrating between 0 and 70 min in order to obtain the graphic area. The area of ROS was standardized by the number of viable cells (Ferreira-Cravo *et al*., 2007).

2.7. Measurement of intracellular reduced glutathione (GSH) concentration

This technique is based on the reactivity of 2,3-naftalenodicarboxialdeido (NDA) to react with GSH to form a cyclic and fluorescent compound. Cells pellets were re-suspended in PBS and then proteins precipitated with sulfosalicylic acid (final concentration: 100 mM). Aliquots of the supernatants were transferred to specific microplates for fluorescence detection. Following it was added to all wells a NDA solution, and after 30 min of incubation, the fluorescence intensity of the NDA-GSH complex was read at excitation and emission wavelength of 485 and 530 nm,

respectively, using a plate reader fluorimeter (Victor 2, Perkin Elmer) (White *et al.*,2003).

2.8. Determination of lipid oxidative damage

The lipid peroxidation was determined by TBARS (Oakes and Van Der Kraak, 2003). This method involves the reaction of malondialdehyde (MDA), a degradation product of lipid peroxidation, with thiobarbituric acid (TBA) under conditions of high temperature and acidity, producing a chromogen which was quantified by fluorometry (excitation: 520 nm, emission: 580 nm). Briefly, after homogenization with PBS, aliquots of hepatocytes were incubated at 95°C during 30 min with 35μM of butylated hydroxytoluene (BHT), 8.1% sodium dodecyl sulfate (SDS), 20% acetic acid and 0.8% TBA. After cooling to room temperature, it was added N-butanol, centrifuged at 3,000 xg for 10 minutes at 15°C. Tetramethoxypropane (ACROS Organics) was used as standard. Fluorescence was read at room temperature using a plate reader fluorimeter (Victor 2, Perkin Elmer).

2.9. Measurement of total antioxidant capacity

Briefly, total antioxidant competence against peroxyl radicals was analyzed through reactive oxygen species (ROS) determination in cells extracts treated or not with a peroxyl radical generator. Peroxyl radicals were produced by thermal (35^{o}C) decomposition of 2,2´-azobis 2 methylpropionamidine dihydrochloride (ABAP; 4 mM; Aldrich) (Winston *et al.,* 1998). Readings were carried out in a fluorescence microplate reader (Victor 2, Perkin Elmer), in a medium containing 30 mM HEPES (pH 7.2), 200 mM KCl, 1 mM $MgCl_{2,}$ 40 μM DCF-DA. Total fluorescence generation (excitation: 485 nm, emission: 530 nm) was calculated by integrating the fluorescence units (FU)

along the measurement time, after adjusting FU data to a second order polynomial function. The relative difference between ROS area with and without ABAP was considered a measure of antioxidant competence, with high area difference meaning low antioxidant capacity, since high fluorescence levels were obtaining after adding ABAP, meaning low competence to neutralize peroxyl radicals (Amado *et al.*, 2009).

2.10. Measurement of glutathione-S-transferase omega (GST Ω) activity

Determination of GST Ω activity was performed by the enzymatic reduction of MM^V, measured spectrophotometrically at 340 nm, resulting from the oxidation of NADPH. The reduction of MMA^V was measured employing 100 µl of cells extracts (made in PBS), 5 mM GSH, 30 µM MM^V, 0.25 mM NADPH, and 0.8 units of glutathione reductase (Sampayo-Reyes and Zakharyan, 2006).

2.11. Measurement of oxidized proteins

Measurement of modified proteins by oxidation (carbonyl groups) was performed using an immunoassay commercial kit (OxyELISA, Millipore) according to manufacturer's instructions. Cells were initially lysed by icing/thawing with a proper buffer supplemented with protease inhibitor phenylmethylsulphonylfluoride (PMSF) and normalized to 10 µg/ml. Aliquots of the lysates were added to a 96-wells micro titer plate and incubated during 1 h at 37°C to adsorb the proteins on the bottom. After this, plate was washed with proper solution and a dinitrophenylhydrazine (DNPH) solution was added to cause derivatization of carbonyl groups of the oxidized proteins side chains, forming 2,4- dinitrophenylhydrazone (DNP-hydrazone) during 45 minutes at 25°C in the dark. Reaction was stopped with a blocking solution. The DNP-derivatized

proteins were then incubated with a mouse monoclonal antibody (conjugated to horseradish peroxidase (HRP)) specific to the DNP moiety during 1 hour at room temperature. After several washes, samples were incubated with the HRP substrate 3,3',5,5'-Tetramethylbenzidine (TMB) and absorbance at 620 nm was monitored in a plate reader spectrophotometer (EL 808, Biotek). Once an optical density of 0.6 to 0.7 was reached (approximately 30 minutes), reaction was stopped by adding a stop solution and samples, and then read at 450 nm. Liquid absorbances of the samples were assumed as a measure of immuno-detection of carbonyl groups present in proteins.

2.12. Measurement of intracellular arsenic accumulation

Before the determination of total As content, cell pellets were digested by using an acidic oxidant mixture containing HNO_3 and H_2O_2. Firstly, a sample amount was weighted, added of 10 mL of concentrated HNO_3 and heated at 95°C for 15 min (without boiling). After cooling, it was added of 5 mL concentrated HNO_3 and refluxed once again for 30 min (95°C). This sample volume was then reduced up to 5 mL and, after cooling, 2 mL of H_2O and 3 mL of H_2O_2 30% were added to the digestion tubes. The solution was heated and added of H_2O_2 30% (1 mL aliquots) until diminishing the solution effervescence. The solution was finally cooled down and diluted to 50 mL with ultrapure water. The determination of As in the digested samples were made by using a calibration curve ranging from 1.0 to 10.0 µg/l of total As. Measurements were performed in a SpectrAA 200 atomic absorption spectrometer (Varian, Australia), equipped with a VGA 77 system for continuous flow hydride generation. The operating conditions of the spectrometer for the determination of arsenic were the following: wavelength 193.7 nm; bandwidth 0.5 nm; lamp current 12 mA. Hydride generation was conducted carrying the reductant, 1% (m/v) sodium tetrahydroborate (III) in 250 mM

NaOH, and 6.0 M HCl, at a flow rate of 1.0 mL/min, and sample at a flow rate of 8.0 mL/min. The atomization temperature was 925°C.

2.13. Statistical analysis

All results were expressed as mean ± standard deviation. Significant differences were assessed by two-factorial analysis of variance (ANOVA), being the factors As^{III} concentration (0; 2.5 or 100 μM) and C_{60} concentration (0 or 1 mg/L).A *posteriori* comparisons were performed by the Newman-Keuls test or by orthogonal contrasts. In all cases, type I error probability was fixed in 5%. ANOVA assumptions (normality and variance homogeneity) were previously checked (Zar, 1984).

3. Results

As showed in **Fig. 1**, fullerene suspensions employed in the assays showed a prevalence of nano-aggregates, as previously reported by Lyon *et al*, (2006) using the same procedure of stirring fullerene in Milli Q water without adding organic solvents. Results of mitochondrial dehydrogenases functionality are represented in **Fig. 2**. The results showed that the treatments did not alter the ability of MTT reduction ($p>0.05$) during the 4 h of exposure to 2.5 or 100 μM As^{III}, both in absence or in presence of fullerene (1 mg/L). Cells co-exposed to arsenic and fullerene registered a significant increase in arsenic concentrations in respect of those treated only with arsenic ($p< 0.05$; **Fig. 3**).

Intracellular ROS concentration in the 100 μM As^{III}+C_{60} (1 mg/L) treatment was significantly lower when compared with the other treatments ($p<0.05$; **Fig. 4**). Also, the

Control+C_{60} (1 mg/L) group presented a lower ROS concentration than the control group without C_{60} ($p<0.05$; **Fig.4**).

Intracellular GSH concentration (**Fig. 5a**) presented a significant increase ($p<0.05$) at the lowest As^{III} concentration (2.5 μM), which was abolished in the presence of C_{60}. Antioxidant capacity data (**Fig. 5b**) presented a significant interaction ($p<0.05$) between the two analyzed factors (arsenic and fullerene exposure). Orthogonal contrasts showed a relative increase ($p<0.05$) in total antioxidant competence against peroxyl radicals in cells exposed to arsenic (both concentrations) and C_{60} when compared with the control group (**Fig. 5b**).

GST Ω activity (**Fig. 6**) showed a significant reduction ($p<0.05$) in cells exposed to 100 μM of As^{III} and co-exposed to C_{60} when compared with cells only exposed to 100 μM of As^{III}. The other treatments did not showed statistical differences ($p>0.05$). TBARS concentration (**Fig. 7a**) was increased ($p<0.05$) at the lowest As^{III} concentration (2.5 μM) when compared with the same As^{III} concentration in presence of C_{60}. Levels of oxidized protein (**Fig.** 7**b)** showed no significant differences ($p>0.05$) between treatments.

4. Discussion

In present study the first step was to conduct assays in order to select concentrations of As^{III} and C_{60} that assured no loss of cell viability. According to the results presented in **Fig. 2**, it was concluded that exposure to arsenite (2.5 and 100μM) without or with fullerene (1 mg/L) co-exposure during 4h caused no significant difference in the ability

to reduce MTT forming formazan, demonstrating that these concentrations were sub-lethal for zebrafish hepatocytes.

When analyzed the oxidative potential of fullerene suspensions, it has been reported that aqueous fullerene suspensions can act as a potent scavenger of ROS, equivalent to the powerful antioxidant BHT and even greater than vitamin E (Torres *et al*., 2011). The results from this study are consistent with those found here, where a clear reduction of intracellular ROS concentration was registered in control+C_{60} treatment when compared to the control group, without fullerene exposure (**Fig. 4**). The antioxidant properties of fullerene have been reported previously. For example, using human neuroblastoma cells exposed to 0.1; 10 or 100 μM of C_{60} during 24 h, it was also observed antioxidant responses, being the fullerene able to scavenge free radicals produced in these cells (Ehrich *et al*., 2010). The term of "radical sponge" was coined for fullerene, in virtue of its scavenging capacity in keratinocytes of human skin irradiated with visible light (400-2.000 nm) (Xiao *et al*., 2006). Our results of total antioxidant capacity also agree with the idea of fullerene being a "radical sponge", as showed by cells co-exposed with arsenite (2.5 and 100 μM) and fullerene (1 mg/L) which presented higher antioxidant capacity (**Fig. 5b**). In fact, co-exposure of C_{60} lowered levels of lipid peroxidation induced at the lowest As concentration (**Fig. 7a**).

When organisms are exposed to conditions that can cause oxidative stress, antioxidant defenses are activated, being GSH one of the main antioxidants involved in the preservation of cellular redox status and defenses against ROS and xenobiotics (White *et al*., 2003). It was reported an increase in GSH levels in zebrafish gills after exposure

of 10 and 100 μg/L of As^{V} (Ventura-Lima *et al*., 2009). In other studied it was analyzed the effect of inorganic arsenic (as sodium arsenite) in human keratinocytes cell line, exposed for 24 h at concentrations as high as 10 μM. It was reported an increased production of hydrogen peroxide, concomitant with higher levels of GSH (Pi *et al*. 2003). In our study, the exposure of zebrafish hepatocytes during 4 h to 2.5 μM of As^{III} was already enough to cause an increase in levels of GSH (**Fig. 5a**). This result indicates a possible oxidant condition elicited by As^{III} in the cell, since GSH is considered an important mediator of the intracellular redox condition. In the 2.5 μM of As^{III}+C_{60} (1 mg/L) treatment, the GSH peak was not observed. As mentioned previously, augmented GSH levels induced by low As concentrations has been previously reported (Pi *et al*., 2003; Xiao *et al.,* 2006), suggesting that the fullerene may be sequestering the metalloid and thus abolishing the GSH peak.

The "Trojan horse" concept implies in a facilitated entry of toxic molecules adsorbed to nanoparticles into the cells (Limbach *et al.,* 2007). As showed in **Fig. 3**, C_{60} in fact favored As accumulation in zebrafish hepatocytes. However, a higher As accumulation when co-exposed with C_{60} did not resulted in higher toxicity. As mentioned above, C_{60} abolished the GSH peak in cells exposed to 2.5 μM of As^{III}, as well as the TBARS peak for the same treatment (**Fig. 7a**). However, it is important to consider if As adsorbed to C_{60} could be then released once inside the cell. For example, it has been analyzed arsenite and arsenate removal efficiency by carbon-based absorbents, being found that different carbon compounds greatly differs in its adsorption capacity, according to the medium pH (Pattanayak*et al*., 2000). From our results it is evident that the existence of "Trojan horse" effect does not imply in toxicity synergy, showing that the kinetics of toxic molecules release from nanoparticles deserves several studies.

When antioxidant defenses are below the level of oxidants, lipid peroxidation can be induced, an event widely accepted as a general mechanism of cellular injury and one of the toxic effects elicited by arsenic (Ramanathan *et al*., 2003; Dhar *et al*., 2005; Flora *et al*.,2005; García-Chávez *et al*., 2006). The increase in lipoid peroxidation at 2.5 μM of As^{III} was in parallel with an increment of GSH levels, an adaptive response that was insufficient to prevent lipid peroxidation. Interestingly, these two effects were not observed in the 2.5 μM of As^{III}+C_{60} (1 mg/L) treatment, indicating that the fullerene may be acting as an antioxidant protecting the cell against lipid damage and/or lowering the intracellular levels of free As, as previously mentioned.

It has been reported that arsenic can cause oxidative damage to proteins due to its high affinity to sulfhydryl groups of proteins (Tseng, 2004). The oxidative damage to proteins is reflected by an increase in the levels of protein carbonyls (PCO), and a decrease in the concentration of protein thiols (PSH). Also, it is known that a by-product of lipid peroxidation as malondialdehyde (MDA) can generate PCO (Samuel *et al*., 2005). Our results showed a peak of TBARS (an estimator of malondialdehyde levels) without being observed significant differences in PCO levels. It is expected that these two parameters of oxidative damage follows a different time course, where a peak in MDA should be the earlier responses and a peak in PCO a later one, not registered with the time frame employed in present study (4 h).

Enzymes of glutathione-S-transferase (GST) group play a key role in cellular detoxification, protecting cells against pollutants or toxicants by conjugating them to glutathione and other endogenous molecules. The GST superfamily has been divided into distinct classes knows as alpha, mu, pi, theta, zeta, sigma and omega (Ω) (Ventura-

Lima *et al*., 2011). The GST Ω 1 form is involved in the reductive step of arsenate (As^V), monomethylarsonic acid (MMA^V), and dimethylarsonic acid (DMA^V). The second form, GST Ω 2, was recently identified and can also catalyze the reduction of monomethylarsonic acid (MMA^V) and dimethylarsonic acid (DMA^V) (Agusa *et al*., 2010). In this way, GST of omega class are crucial enzymes in the pathway for the reduction of arsenate to arsenite in the methylation pathway (Chowdhury *et al*., 2006).We observed a reduced GST Ω activity in the treatment 100 μM of As^{III}+C_{60} (1 mg/L), when compared with the treatment of 100 μM of As^{III}, showing that arsenic alone provides higher capacity for arsenic methylation. The lowering of GST Ω activity when As is co-administered with C_{60} raises again the idea of arsenic being trapped to fullerene, thus abolishing this enzymatic response. Also it raises the question about fullerene influence in As methylation pathway, a point that urge to be analyzed.

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References

Agusa, T., Iwata, H., Fujihara, J., Kunito, T., Takeshita, H., Minh, T.B., Trang, P.T.K.,Viet, P.H., Tanabe, S. 2010. Genetic polymorphisms in glutathione S-transferase (GST) superfamily and arsenic metabolism in residents of the Red River Delta, Vietnam. Toxicol. Appl. Pharmacol. 242, 352–362.

Amado, L.L., Garcia, M.L., Ramos, P.B., Freitas, R.F., Zafalon, B., Ferreira, J.L.R., Yunes, J.S., Monserrat, J.M., 2009. A method to measure total antioxidant capacity against peroxyl radicals in aquatic organisms: Application to evaluate microcystins toxicity. Sci. Tot. Environ. 407, 2115-2123.

Aschberger K., Johnston H.J., Stone V., Aitken R., Tran L.C., Hankin S., Peters S.,Christensen F., 2010. Review of fullerene toxicity and exposure – Appraisal of a human health risk assessment, based on open literature. Regul. Toxicol. Pharmacol. 58, 455- 473.

Chowdhury, K.U, Zakharyan, R.A., Hernandez, A., Avram, M.D., Kopplin, M. J., Aposhian, H.V., 2006. Glutathione-S-transferase-omega [MMA(V) reductase] knockout mice: Enzyme and arsenic species concentrations in tissues after arsenate administration. Toxicol. Appl. Pharmacol. 216, 446–457.

Chowdhury, R., Chatterjee, R. Giri, A.K., Mandal, C., Chaudhuria, K., 2010. Arsenic-induced cell proliferation is associated with enhanced ROS generation, Erk signaling and Cyclin A expression. Toxicol. Lett. 198, 263–271.

Delnomdedieu, M., Basti, M.M., Otvos, J.D., Thomas, D.J., 1994. Reduction and binding of arsenate and dimethylarsinate by glutathione: a magnetic resonance study. Chem. Biol. Interact. 90, 139-155.

Dhar, P., Jaitley, M., Kalaivani, M., Mehra, R.D., 2005. Preliminary morphological and histochemical changes in rat spinal cord neurons following arsenic ingestion. Neurotoxicol. 26, 309–20.

Ehrich, M., Tassell, R. V., Li, Y., Zhou, Z, Kepley, C.L., 2010. Fullerene antioxidants decrease organophosphate-induced acetylcholinesterase inhibition in vitro. Toxicol. in Vitro. 25, 301- 307.

Ferreira-Cravo, M., Piedras, F.R., Moraes, T.B., Ferreira, J.L.R., Freitas, D.P.S., Machado, M.D., Geracitano, L.A., Monserrat, J.M., 2007. Antioxidant responses and reactive oxygen species generation in different body regions of the estuarine polychaeta *Laeonereis acuta* (Nereididae), Chemosphere. 66, 1367-1374.

Fischer, H.C., Chan, C.W., 2007. Nanotoxicity: the growing need for in vivo study. Curr. Opin. Biotechnol. 18, 565–571.

Flora, S.J.S., Bhadauria, S., Pant, S.C., Dhaked, R.K., 2005. Arsenic induced blood and brain oxidative stress and its response to some thiol chelators in rats. Life Sci. 77, 2324–37.

García-Chávez, E., Jiménez, I., Segura, B., Del Razo, L.M., 2006. Lipid oxidative damage and distribution of inorganic arsenic and its metabolites in the rat nervous system after arsenite exposure: Influence of alpha tocopherol supplementation, Neurotoxicol. 27, 1024–1031.

Gharbi, N., Pressac, M., Hadchouel, M., Szwarc, H., Wilson, S.R., Moussa, F., 2005. Fullerene is a powerful antioxidant in vivo with no acute or sub-acute toxicity. Nano Lett. 5, 2578-2585.

Henry, T.B., Menn, F.M., Fleming, J.T., Wilgus, J., Compton, R.N., Sayler, G.S., 2007. Attributing effects of aqueous C_{60} nano-aggregates to tetrahydrofuran decomposition products in larval zebrafish by assessment of gene expression. Environ. Health Perspect. 115, 1059-1065.

Kahru, A., Dubourguier, H.C., 2010. From ecotoxicology to nanoecotoxicology. Toxicology. 269, 105-119.

Kamat, P.V., 2002. Photophysical, photochemical and photocatalytic aspects of metal nanoparticles. Am. Chem. Soc. 106, 7729–7744.

Limbach, L.K., Peter, W., Pius, M., Grass, R.N., Bruinink, A., Stark, W.J., 2007. Exposure of engineered nanoparticles to human lung epithelial cells: Influence of chemical composition and catalytic activity on oxidative stress. Environ. Sci. Technol. 41, 4158–4163.

Lyon, D.Y., Adams, L.K., Falkner, J.C., Alvarez, P.J.J., 2006. Antibacterial activity of fullerene water suspensions: effects of preparation method and particle size. Environ. Sci. Technol. 40, 4360-4366.

Mori, T., Takada, H., Ito, S., Matsubayashi, K., Miwa, N., Sawaguchi, T., 2006. Preclinical studies on safety of fullerene upon acute oral administration and evaluation for no mutagenesis. Toxicology. 225, 48–54.

Oakes, K.D., Van Der Kraak, G.J., 2003. Utility of the TBARS assay in detecting oxidative stress in white sucker (*Catostomus commersoni*) populations exposed to pulp mill effluent. Aquatic. Toxicol. 63, 447- 463.

Oberdörster, E., 2004. Manufactured nanomaterials (fullerenes, C_{60}) induce oxidative stress in the brain of juvenile largemouth bass. Environ. Health Perspect. 112, 1058-1062.

Oberdörster, G., Oberdörster, E., Oberdörster, J., 2005. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ. Health Perspect. 113, 823-839.

Oberdörster, E., Zhu, S., Blickley, M., McClellan-Green, P., Haasch, M.L., 2006. Ecotoxicology of carbon-based engineered nanoparticles: Effects of fullerene (C_{60}) on aquatic organisms. Carbon. 44, 1112-1120.

Oberdörster, G., Stone, V., Donaldson, K., 2007. Toxicology of nanoparticles: a historical perspective. Nanotoxicol. 1, 2-25.

Pattanayak, J., Mondal, K., Mathew, S., Lalvani, S.B., 2000. A parametric evaluation of the removal of As(V) and As(III) by carbon-based adsorbents. Carbon. 38, 589–596.

Pi, J., Qu, W., Reece, J.M., Kumagai, Y., Waalkes, M.P., 2003.Transcription factor Nrf2 activation by inorganic arsenic in cultured keratinocytes: Involvement of hydrogen peroxide. Exp.Cell Res. 290, 234–245.

Porter, A.E., Gass, M., Muller, K., Skepper, J.N., Midgley, P., Welland, M., 2007. Visualizing the uptake of C_{60} to the cytoplasm and nucleus of human monocyte-derived macrophage cells using energy-filtered transmission electron microscopy and electron tomography. Environ. Sci. Technol. 41, 3012–3017.

Ramanathan, K., Shila, S., Kumaran, S., Panneerselvan, C., 2003 . Ascorbic acid and α-tocopherol as potent modulators on arsenic induced toxicity in mitochondria. J. Nutr. Biochem. 14, 416-420.

Samuel S, Kathirvel, R., Jayavelu, T., Chinnakkannu, P., 2005. Protein oxidative damage in arsenic induced rat brain: influence of DL-α-lipoic acid. Toxicol. Lett. 155, 27–34.

Sampayo-Reyes, A., Zakharyan, R.A., 2006. Inhibition of human glutathione-S-transferase omega by tocopherol succinate. Biomed. Pharmacother. 60, 238-244.

Sakurai, T., Ochiai, M., Kojima, C., Ohta, T., T., Sakurai, M.H., Takada, N.O., Qu, W., Wallkes, M.P., Himeno, S., Fujiwara, K., 2005. Preventive mechanism of cellular glutathione in monomethylarsonic acid-induced cytolethality. Toxicol. Appl. Pharmacol. 206, 54-65.

Schuliga, M., Chouchane, S., Snow, E.T., 2002. Up-regulation of glutathione-related genes and enzymes activities in cultured human cells by sub-lethal concentration of inorganic arsenic. Toxicol. Sci. 70, 183-192.

Seok, S.H., Baek, M.W., Lee, H.Y., Kim, D.J., Na, Y.R., Noh, K.J., Park, S.H., Lee, H.K., Lee, B.H., Ryu D.Y., Park, J.H., 2007. Arsenite induced apoptosis is prevented by antioxidants in zebrafish liver cell line. Toxicol in Vitro. 21, 870–877.

Shinohara, N., Matsumoto, K., Endoh, S., Maru, J., Nakanishi, J., 2009. In vitro and in vivo genotoxicity tests on fullerene C_{60} nanoparticles. Toxicol. Lett. 191, 289-296.

Sun, H., Zhang, X., Zhang, Z., Chen, Y., Crittenden, J.C., 2009. Influence of titanium dioxide nanoparticles on speciation and bioavailability of arsenite. Environ. Pollut. 157, 1165–1170.

Thomas, D.J., Styblo, M., Lin, S., 2001. The cellular metabolism and systemic toxicity of arsenic. Toxicol. Appl. Pharmacol. 176, 127-144.

Torres, V.M., Posac M., Srdjenovi, B., Simplicio, A.L., 2011. Solubilization of fullerene C_{60} in micellar solutions of different solubilizers. Colloids and Surfaces B: Biointerfaces. 82, 46–53.

Tseng, C., 2004. The potential biological mechanism of arsenic-induced diabetes mellitus. Toxicol. Appl. Pharmacol. 197, 67-83.

Usenko, C.Y., Harper, S.L., Tanguay, R.L., 2007. In vivo evaluation of carbon fullerene toxicity using embryonic zebrafish. Carbon. 45, 1891-1898.

Usenko, C.Y., Stacey, L.H., Tanguay, R.L., 2008. Fullerene C_{60} exposure elicits an oxidative stress response in embryonic zebrafish. Toxicol. Appl. Pharmacol. 229, 44-55.

Ventura-Lima, J., Fattorini, D., Regoli, F., Monserrat, J.M., 2009. Effects of different inorganic arsenic species in *Cyprinus carpio* (Cyprinidae) tissues after short-time exposure: Bioaccumulation, biotransformation and biological responses. Environ.Pollut. 157, 3479–3484.

Ventura-Lima, J., Bogo, R.B., Monserrat, J.M., 2011. Arsenic toxicity in mammals and aquatic animals: A comparative biochemical approach. Ecotoxicol. Environ. Saf. 74, 211-218.

Xiao, L., Takada, H., Gan, X.H., Miwa, N., 2006 .The water-soluble fullerene derivative 'radical sponge' exerts cytoprotective action against UVA irradiation but not visible-light-catalyzed cytotoxicity in human skin keratinocytes. Bioorg. Med. Chem. Lett. 16, 1590–1595.

Xuexia, Z., Yongfeng, J., Xin, W., Liying, X., 2008. Phylogenetic analysis and arsenate reduction effect of the arsenic-reducing bacteria enriched from contaminated soils at an abandoned smelter site. J. Environ. Sci. 20, 1501–1507.

Zar, J. H., 1984. Biostatistical Analysis, 2nd. ed. Ed. Prentice Hall, New Jersey.

Zhu, S., Oberdörster, E., Haasch, M., 2006. Toxicity of an engineered nanoparticle (fullerene, C_{60}) in two aquatic species, *Daphnia* and fathead minnow. Mar. Environ. Res. 62, S5-S9.

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

Wang, H., Joseph, J.A., 1999. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Rad. Biol. Med. 27, 612-616.

White, C.C., Viernes, H., Krejsa, C.M., Botta, D., Kavanagh, T.J., 2003. Fluorescence-based microtiter plate assay for glutamate-cysteine ligase activity. Anal. Biochem. 318, 175-180.

Winston, G.W., Regoli, F., Dugas, Jr. A.J., Fong, J.H., Blanchard, K.A., 1998. A rapid gas chromatographic assay for determining oxyradical scavenging capacity of antioxidants and biological fluids. Free Rad. Biol. Med. 24, 480-493.

Figures captions

Figure 1. Transmission electronic microscopy (TEM) image of the aqueous fullerene (C_{60}) suspensions employed in the bioassays.

Figure 2. Number of viable zebrafish hepatocytes after exposure during 4 h to 0 (control); 2.5; or 100 μM As^{III} with or without fullerene (1 mg/L). Numbers of analyzed samples were 4 for each treatment, being conducted three independent experiments. Identical letters means absence of statistical differences ($p > 0.05$).

Figure 3. Arsenic accumulation (μg per cell) by zebrafish hepatocytes after exposure during 4 h to 0 (control); 2.5; or 100 μM As^{III} with or without fullerene (1 mg/L). Numbers of analyzed samples were 4 for each treatment, being conducted two independent experiments. Identical letters means absence of statistical differences ($p > 0.05$).

Figure 4. Intracellular reactive oxygen species (ROS) concentration in zebrafish hepatocytes after exposure during 4 h to 0 (control); 2.5; or 100 μM As^{III} with or without fullerene (1 mg/L). Numbers of analyzed samples were 4 for each treatment, being conducted three independent experiments. Identical letters means absence of statistical differences ($p > 0.05$).

Figure 5. (a). Intracellular concentration of reduced glutathione (GSH) (nM/mg of proteins) in hepatocytes of zebrafish, after exposure during 4 h to 0 (control); 2.5; or 100 μM As^{III} with or without fullerene (1 mg/L). Numbers of analyzed samples were 4

for each treatment, being conducted three independent experiments. Identical letters means absence of statistical differences ($p > 0.05$). **(b)**. Same as **(a)** for total antioxidant capacity against peroxyl radicals (relative area). Asterisk (*) indicates significant differences ($p < 0.05$) between treatments connected by solid lines.

Figure 6. Activity of glutathione-S-transferase (GST) omega (nmol NADPH/min/mg of proteins) in hepatocytes of zebra fish after exposure during 4 h to 0 (control); 2.5; or 100 μM As^{III} with or without fullerene (1 mg/L). Numbers of analyzed samples were 4 for each treatment, being conducted three independent experiments. Asterisk (*) indicates significant differences ($p < 0.05$) between treatments connected by solid lines.

Figure 7. (a) Intracellular concentration of thiobarbituric acid reactive substances (TBARS; nmol/mg of proteins) in hepatocytes of zebra fish after exposure during 4 h to 0 (control); 2.5; or 100 μM As^{III} with or without fullerene (1 mg/L). Numbers of analyzed samples were 4 for each treatment, being conducted three independent experiments. **(b)**. Same as (a) for oxidized proteins. Identical letters means absence of statistical differences ($p > 0.05$). In **(a)** and **(b)**, asterisk (*) indicates significant differences ($p < 0.05$) between treatments connected by solid lines.

Figure 1

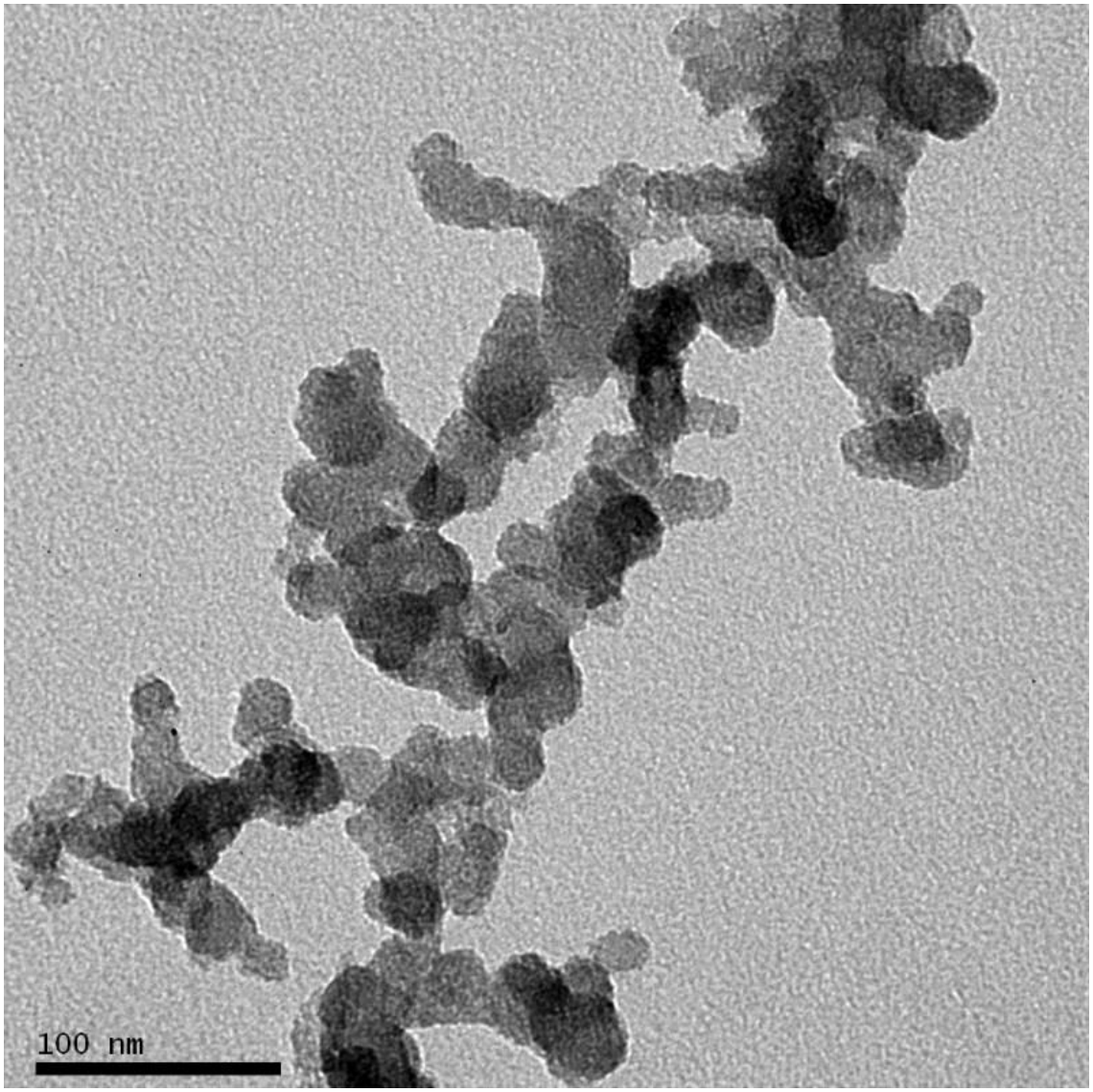


Figure 2

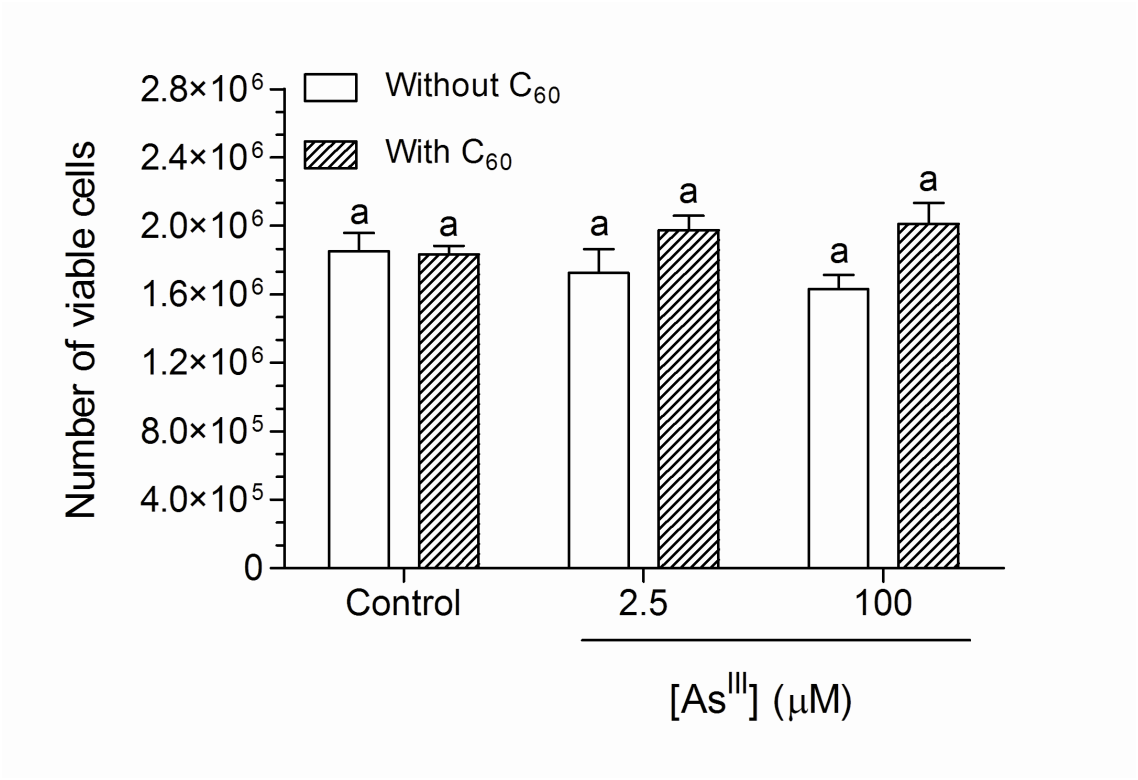

Number of viable cells
2.8×10^6
2.4×10^6
2.0×10^6
1.6×10^6
1.2×10^6
8.0×10^5
4.0×10^5
0
Without C60
With C60
a
a
a
a
a
a
Control
2.5
100
[As^III] (μM)

Figure 3

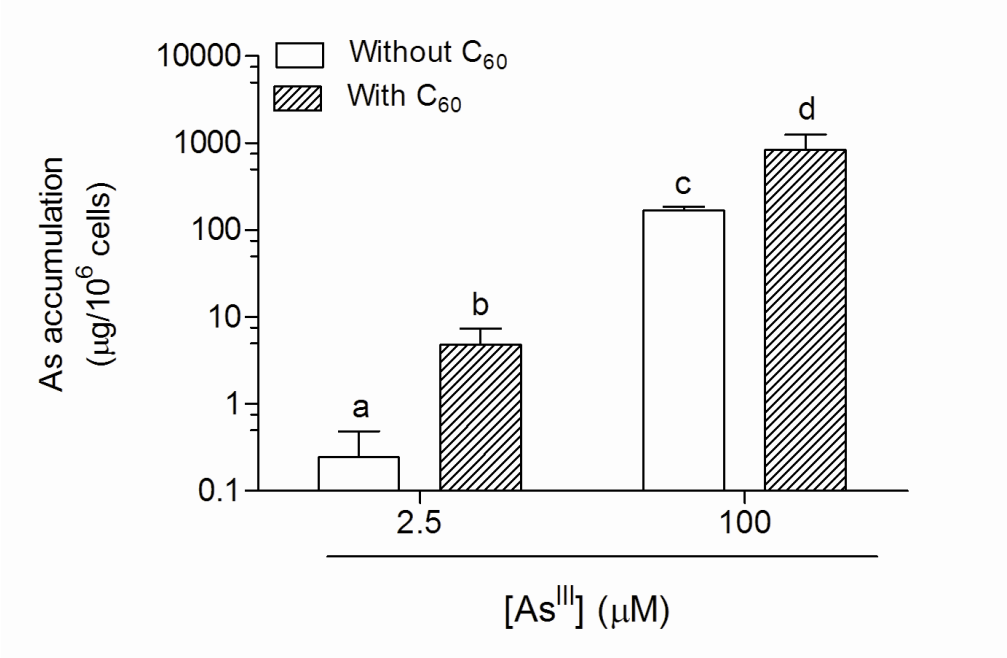

10000
1000
100
10
1
0.1
As accumulation
(μg/10^6 cells)
Without C60
With C60
a
b
c
d
2.5
100
[As^III] (μM)

Figure 4

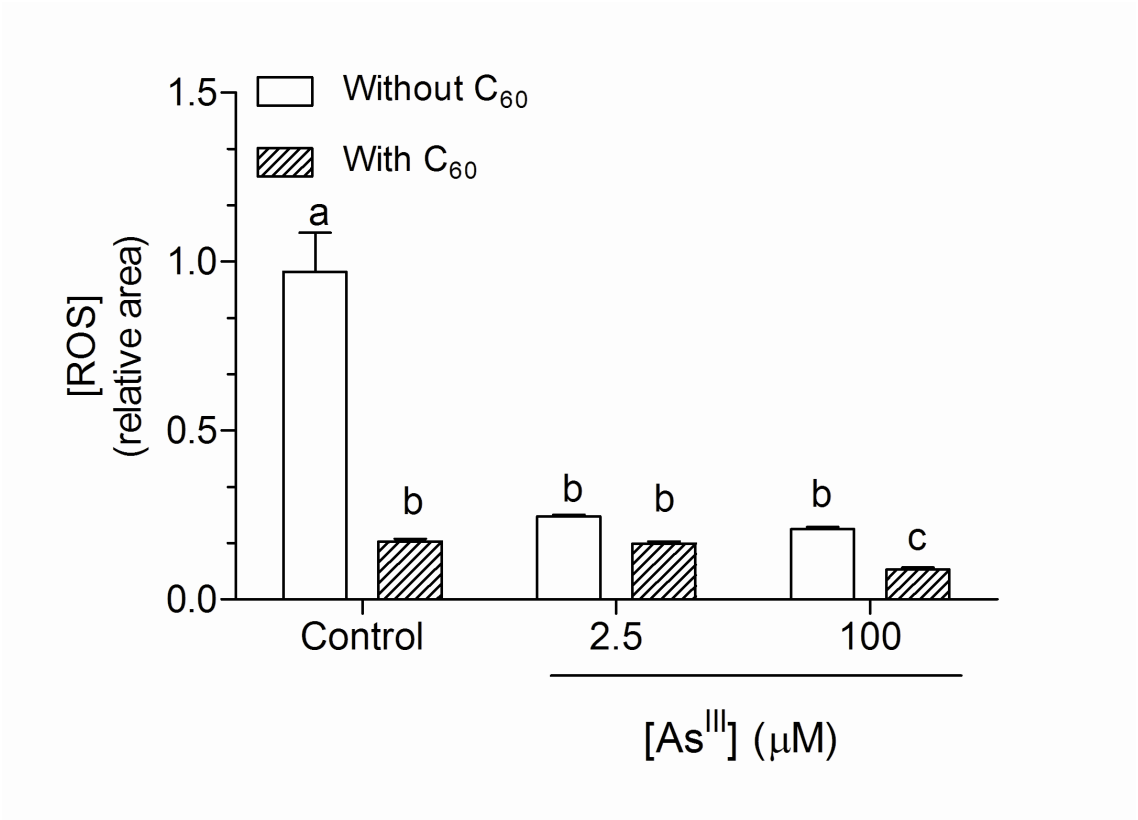

Without C_{60}
With C_{60}
[ROS]
(relative area)
1.5
1.0
0.5
0.0
a
b
b
b
b
c
Control
2.5
100
[As^{III}] (μM)

Figure 5

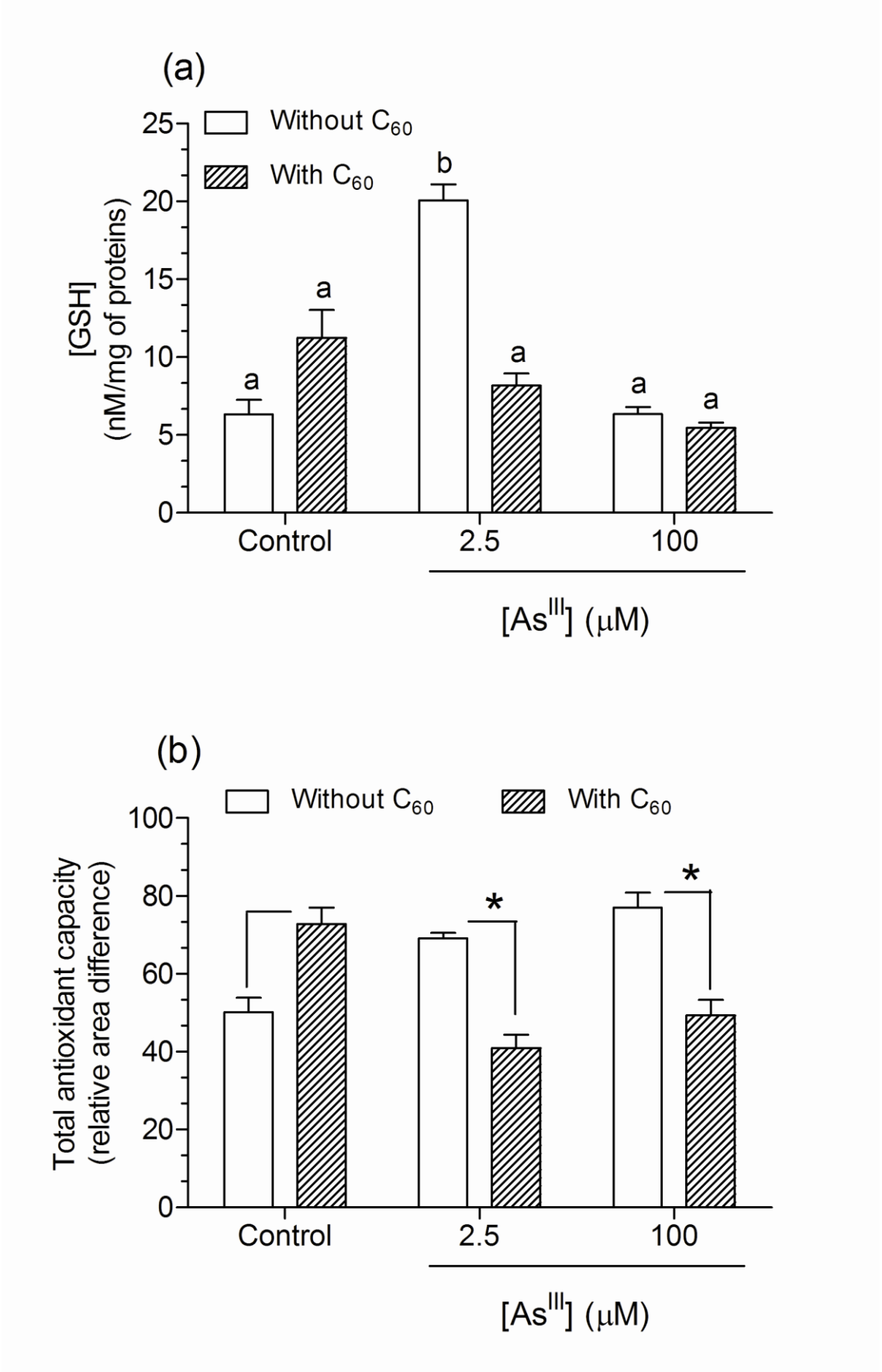

(a)
Without C_{60}
With C_{60}
[GSH] (nM/mg of proteins)
25
20
15
10
5
0
a
a
b
a
a
a
Control
2.5
100
[As^{III}] (μM)
(b)
Without C_{60}
With C_{60}
Total antioxidant capacity (relative area difference)
100
80
60
40
20
0
*
*
Control
2.5
100
[As^{III}] (μM)

Figure 6

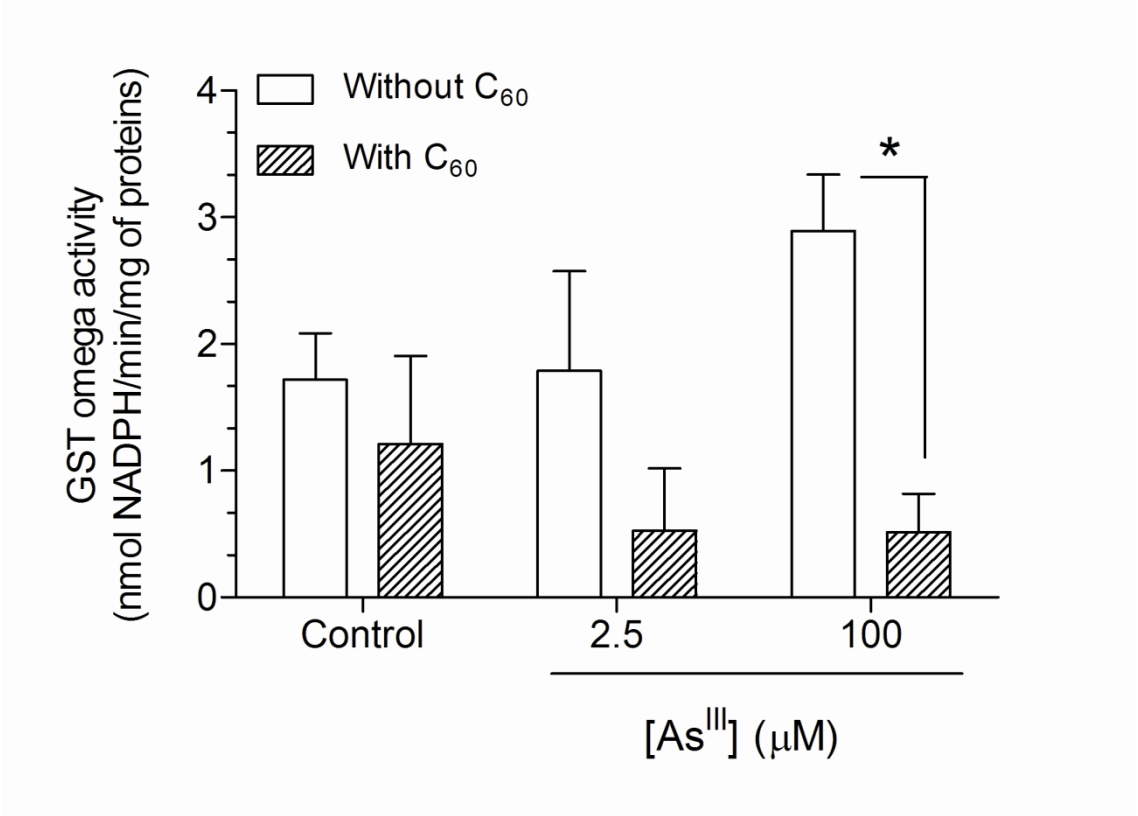
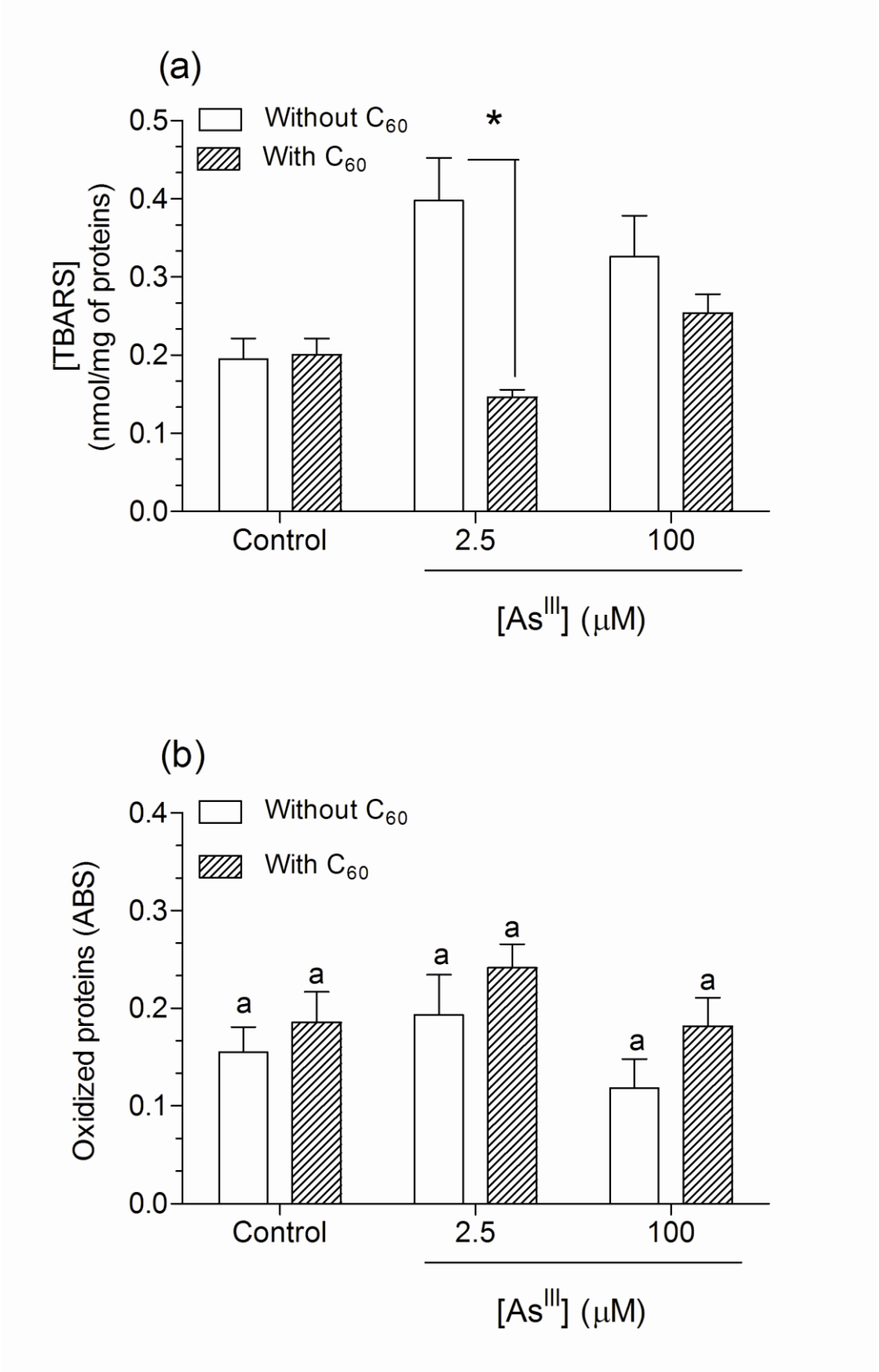

GST omega activity
(nmol NADPH/min/mg of proteins)
Without C_{60}
With C_{60}
*
0
1
2
3
4
Control
2.5
100
[As^{III}] (μM)

Figure 7


(a)
Without C_{60}
With C_{60}
*
[TBARS] (nmol/mg of proteins)
0.0
0.1
0.2
0.3
0.4
0.5
Control
2.5
100
[As^{III}] (μM)
(b)
Without C_{60}
With C_{60}
Oxidized proteins (ABS)
0.0
0.1
0.2
0.3
0.4
a
a
a
a
a
a
Control
2.5
100
[As^{III}] (μM)

CONCLUSÕES

No presente estudo com a analise das diferentes variáveis bioquímicas, pode-se concluir que o fulereno teve a capacidade de reduzir a produção de espécies ativas de oxigênio nas amostras do grupo controle e na concentração de 100 As μM, reduzindo também a atividade da enzima antioxidante GST Ω na maior concentração de arsênio, e diminuindo a concentração do antioxidante não enzimático GSH na menor concentração de arsênio. Esses resultados estão correlacionados com os dados de co-exposição da menor concentração de As com C_{60}onde houve redução significativa na peroxidação lipídica, e também com o resultado das proteínas oxidadas, onde não houve diferenças significativas após a mesma exposição. Todos os resultados encontrados neste trabalho são fortemente sustentados pela comprovação do papel de carreadores das nanopartículas, pois a concentração intracelular de arsênio nas células co-expostas com fulereno apresentou um resultado significativamente maior. Futuros estudos são necessários para avaliar a biodisponibilidade intracelular de arsênio depois de ser ligado ao nanomaterial fulereno. Portanto conclui-se que nestas concentrações, durante o tempo de exposição e com o modelo experimental utilizado o nanomaterial fulereno co-exposto com o arsênio apresenta capacidade antioxidante.

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REFERÊNCIAS

Agusa,T., Iwata,H., Fujihara, J., Kunito,T., Takeshita,H., Minh,T.B., Trang,P.,T.,K., Viet,P.H., Tanabe, S., Genetic polymorphisms in glutathione S-transferase (GST) superfamily and arsenic metabolism in residents of the Red River Delta, Vietnam. *Toxicology and Applied Pharmacology* **242**, 352–362 (2010)

Akter, K.F.,Owens,G.,Davey,D.E.,Naidu,R., Arsenic speciation and toxicity in biological systems. *Rev. Environ. Contam. Toxicol*. **184**, 97–149 (2005)

Bagnyukova, T.V.,Luzhna,L.I.,Pogribny,I.P.,Lushchak,V.I., Oxidative stress and antioxidant defenses in gold fish liver in response to short-term exposure to arsenite. *Environ. Mol. Mut*. **48**, 658–665 (2007)

Bhattacharya, A., Bhattacharya, S. Induction of oxidative stress by arsenic in *Clarias batracus*: Involvement of peroxisomes. *Ecotoxicology and Environmental Safety* **66**, 178-187 (2007)

Bhatt, I., Tripathi, B.N. Interaction of engineered nanoparticles with various components of the environment and possible strategies for their risk assessment. *Chemosphere* **82**, 308-317 (2010)

Blickley, T., M., Mcclellan-Green, P. Toxicity of aqueous fullerene in adult and larval *Fundulus heteroclitus*. *Environmental Toxicology and Chemistry* **27**, 1964–1971 (2008)

Farkas, J., Christian, P., Urrea, J.A.G., Roos, N., Hassellöv, M., Tollefsen, K.E., Thomas, K. Effects of silver and gold nanopartículas on rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aquat.Toxicol*. **96**, 44-52 (2010)

Galoway, T., Lewis, C., Dolciotti, I., Johnston, B.D., Moger, J., Regoli. Sub-lethal toxicity of nano-titanium and carbon nanotubes in a sediment dwelling marine polychaeta. *Environ. Poll*. **158**, 1748-1755 (2010)

Gharbi, N., Pressac, M., Hadchouel, M., Szwarc, H., Wilson, S.R., Moussa, F. Fullerene is a powerful antioxidant in vivo with no acute or sub-acute toxicity, *Nano Letters* **5**, 2578-2585 (2005)

Hamilton, E.I. Environmental variables in a holistic evaluation of land contaminated by historic mine wastes: a study of multi-element mine wastes in West Devon, England using arsenic as an element of potential concern to human health. *Science of the Total Environment* **249**, 171-221 (2000)

Hughes, M.F. Arsenic toxicity and potential mechanisms of action. *ToxicologyLetters*, **133**, 1–16 (2002)

Kahru, A., Dubourguier, H.C. From ecotoxicology to nanoecotoxicology. *Toxicology* **269**, 105-119 (2010)

Kamat, J.P., Devasagayam, T.P.A., Priyadarsini, K.I., Mohan, H., Mittal, J. P. Oxidative damage induced by the fullerene C_{60} on photosensitization in rat liver microsomes. *Chemico-Biological Interactions* **114**, 145-159 (1998)

Kroto, H.W. Supramolecular chemistry of fullerenes.*Tetrahedron* **62**, 1921-1927 (2006)

Laparra, J.M., Velez, D., Barbera, R., Farre, R., Montoro, R. As2O3-induced oxidative stress and cycle progression in a human intestinal epithelial cell line (Caco-2). *Toxicology in Vitro* **22**, 444–449 (2008)

Limbach, L.K., Peter, W., Pius, M., Grass, R.N., Bruinink, A., Stark, W.J. Exposure of engineered nanoparticles to human lung epithelial cells: Influence of chemical composition and catalytic activity on oxidative stress. *Environmental Science and Technology* **41**, 4158–4163 (2007)

Loutfy, R.O., Lowe, T.P., Moravsky, A.P., Katagiri, S. *Perspectives of fullerene nanotechnology*.Netherlands: Springer; *Commercial production of fullerenes and carbon nanotubes*; pp. 35–46 (2002)

Mandal, B.K., Suzuki, K.T. Arsenic round the world: A review. *Talanta* **58**, 201-235 (2002)

Mirlean, N., Roisenberg, A. The effect of emissions of fertilizer production on the environment contamination by cadmium and arsenic in southern Brazil. *Environmental Pollution* **143**, 335-340 (2006)

Mori, T., Takada, H., Ito, S., Matsubayashi, K., Miwa, N., Sawaguchi, T. Preclinical studies on safety of fullerene upon acute oral administration and evaluation for no mutagenesis. *Toxicology* **225**, 48–54 (2006)

Oberdörster, E. Manufactured nanomaterials (fullerenes, C_{60}) induce oxidative stress in the brain of juvenile largemouth bass. *Environmental Health Perspectives* **112**, 1058-1062 (2004)

Oberdörster, G., Oberdörster, E., Oberdörster, J. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. *Environmental Health Perspectives* **113**, 823-839 (2005)

Oberdörster, G., Stone, V., Donaldson, K. Toxicology of nanoparticles: a historical perspective. *Nanotoxicology* **1**, 2-25 (2007)

Ochi, T. Arsenic compound-induced increase in glutathione levels in cultured Chinese hamster V79 cells and mechanism associated with changes in γ-glutamylcysteine syntetase activity, cysteine uptake and utilization of cysteine. *Archives of Toxicology* **71**, 730-740 (1997)

Popovic, A., Djordjevic, D., Polic, P. Trace and major element pollution originating from coal ash suspension and transport processes. *Environmental International* **26**, 251-255 (2001)

Porter, A.E., Gass, M., Muller, K., Skepper, J.N., Midgley, P., Welland, M. Visualizing the uptake of C_{60} to the cytoplasm and nucleus of human monocyte-derived macrophage cells using energy-filtered transmission electron microscopy and electron tomography. *Environmental Science* & *Technology* **41**, 3012–3017 (2007)

Ramanathan, K., Shila, S., Kumaran, S., Panneerselvan, C. Ascorbic acid and α-tocopherol as potent modulators on arsenic induced toxicity in mitochondria. *Journal of Nutritional Biochemistry* **14**, 416-420 (2003)

Sakurai, T., Ochiai, M., Kojima, C., Ohta, T.T., Sakurai, M.H., Takada, N.O., Qu, W., Wallkes, M.P., Himeno, S., Fujiwara, K. Preventive mechanism of cellular glutathione in monomethylarsonic acid-induced cytolethality. *Toxicology and Applied Pharmacology* **206**, 54-65 (2005)

Sayes, C.M., Gobin, A.M., Ausman,.KD., Mendez, J., West, J.L., Colvin, V.L. Nano-C_{60} cytotoxicity is due to lipid peroxidation. *Biomaterials* **26**, 7587-7595 (2005)

Sharma,V.K., Sohn, M. Aquatic arsenic: Toxicity, speciation, transformations, and remediation. *Environment International* **35**, 743–759 (2009)

Shila, S., Ramanathan, K., Yayavelu, T., Chinnakkannu, P. Protein oxidative damage in arsenic induced rat brain: influence of DL-α-lipoic acid. *Toxicology Letters* **155**, 27-34 (2005)

Shinohara, N., Matsumoto, K.., Endoh, S., Maru, J., Nakanishi, J. In vitro and in vivo genotoxicity tests on fullerene C_{60} nanoparticles. *Toxicology Letters* **191**, 289-296 (2009)

Sayes C.M., Gobin A.M., Ausman, K.D., Mendez, J., West, J.L., Colvin, V.L. Nano-C_{60} cytotoxicity is due to lipid peroxidation. *Biomaterials* **26**, 7587-7595 (2005)

Sun, Z.H., Zhang, X., Zhang, Z., Chen, Y., Crittenden, J.C. Influence of titanium dioxide nanoparticles on speciation and bioavailability of arsenite. *Environmental Pollution* **157**, 1165–1170 (2009)

Tseng, C. The potential biological mechanism of arsenic-induced diabetes mellitus. *Toxicology and Applied Pharmacology* **197**, 67-83 (2004)

Usenko, C.Y., Harper, S.L., Tanguay, R.L. In vivo evaluation of carbon fullerene toxicity using embryonic zebrafish.*Carbon* **45**, 1891-1898 (2007)

Ventura-Lima, J., Fattorini, D., Regoli, F., Monserrat, J.M. Effects of different inorganic arsenic species in *Cyprinus carpio* (Cyprinidae) tissues after short-time exposure: Bioaccumulation, biotransformation and biological responses. *Environmental Pollution* **157**, 3479–3484(2009a)

Ventura-Lima, J., Castro, M.R., Acosta, D., Fattorini, D., Regoli, F., Carvalho, L.M., Boher, D., Geracitano, L.A., Barros, D.M., Marins, L.F., Silva, R.S., Bonan, D.C., Bogo, M.R., Monserrat, J.M. Effects of arsenic (As) exposure on the antioxidant status

of gills of the zebrafish *Danio rerio* (Cyprinidae). *Comparative Biochemistry and Physiology* **C149**, 538-543 (2009b)

Ventura-Lima, J., Bogo, R.B. Monserrat, J.M. Arsenic toxicity in mammals and aquatic animals: A comparative Biochemical approach. *Ecotoxicology and Environmental Safety*, **74**, 211-218 (2011)

Wang, H., Joseph, J.A. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radical Biology & Medicine* **27**, 612-616 (1999)

Wang, Y.C., Chaung, R.H., Tung, L.C. Comparison of the cytolethality induced by different exposure to sodium arsenite in two fish cell lines. *Aquatic Toxicology* **69**, 67-79 (2004)

White, C.C., Viernes, H., Krejsa, C.M., Botta, D., Kavanagh, T.J. Fluorescence-based microtiter plate assay for glutamate-cysteine ligase activity. *Analytical Biochemistry* **318**, 175-180 (2003)

Xu, Y., Wang, H., Wang, Y., Zheng, Y., Sun, G. Effects of folate on arsenic toxicity in Chang human hepatocytes: Involvement of folate antioxidant properties. *Toxicology Letters* **195**, 44–50 (2010)

Zhu, X., Zhu, L., Land, Y., Chen, Y. Oxidative stress and growth inhibition in the freshwater fish *Carassius auratus* induced by chronic exposure to sub-lethal fullerene aggregates. *Environmental Toxicology and Chemistry* **27**, 1979–1985 (2008)