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EFEITOS DO DISSELENETO DE DIFENILA SOBRE O DANO OXIDATIVO CAUSADO POR PARACETAMOL EM CÉREBRO DE CAMUNDONGOS

DISSERTAÇÃO DE MESTRADO

Michele Hinerasky da Silva

Santa Maria, RS, Brasil

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EFEITOS DO DISSELENETO DE DIFENILA SOBRE O DANO OXIDATIVO CAUSADO POR PARACETAMOL EM CÉREBRO DE CAMUNDONGOS

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Orientador: Prof. Dr. Félix Alexandre Antunes Soares

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EFEITOS DO DISSELENETO DE DIFENILA SOBRE O DANO OXIDATIVO CAUSADO POR PARACETAMOL EM CÉREBRO DE CAMUNDONGOS

elaborada por
Michele Hinerasky da Silva

como requisito parcial para obtenção do grau de
Mestre em Ciências Biológicas: Bioquímica Toxicológica

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O único lugar onde o sucesso vem antes do trabalho é no dicionário.

(Albert Einstein)

RESUMO

Dissertação de Mestrado

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica

Universidade Federal de Santa Maria, RS, Brasil

EFEITOS DO DISSELENETO DE DIFENILA SOBRE O DANO OXIDATIVO CAUSADO POR PARACETAMOL EM CÉREBRO DE CAMUNDONGOS

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ORIENTADOR: FÉLIX ALEXANDRE ANTUNES SOARES

Local e Data da Defesa: Santa Maria, 11 de junho de 2012.

O paracetamol é o analgésico mais usado no mundo, em doses terapêuticas não apresenta nenhuma toxicidade, porém em doses elevadas pode causar dano hepático pela formação do seu metabólito tóxico *N*-acetil-*p*-benzoquinoneimina (NAPQI). O dano cerebral pode ocorrer em decorrência ao dano no fígado, uma condição chamada de encefalopatia hepática, já que o fígado pode apresentar um comprometimento das suas funções, como transformar amônia em uréia, causando um acúmulo de amônia no organismo, sendo esta tóxica para o cérebro. Além disso, o NAPQI pode causar estresse oxidativo e disfunção mitocondrial no cérebro. O disseleneto de difenila [$(PhSe)_2$] é um composto orgânico de selênio que apresenta atividade antioxidante e potencial farmacológico. O objetivo desse estudo é verificar a capacidade do $(PhSe)_2$ em reverter o dano cerebral e disfunção mitocondrial causada por uma dose tóxica de paracetamol. Os camundongos receberam paracetamol (600 mg/kg) e 1h após, disseleneto de difenila (15,6 mg/kg). Após 4h da administração do paracetamol, coletou-se o soro para as análises bioquímicas de aspartato aminotransferase (AST) e alanina aminotransferase (ALT) que confirmaram o dano hepático. A administração de APAP aumentou a peroxidação lipídica, a produção de espécies reativas de oxigênio e diminuiu a atividade da enzima Na^+, K^+ - ATPase. Da mesma forma, o APAP alterou os parâmetros de funcionalidade mitocondrial. O tratamento com $(PhSe)_2$ reverteu o dano cerebral induzido por uma dose única de APAP.

Palavras-Chave: estresse oxidativo, disfunção mitocondrial, dano hepático, antioxidante

ABSTRACT

Master Dissertation
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EFFECTS OF DIPHENYL DISSELENIDE ON OXIDATIVE DAMAGE INDUCED BY ACETAMINOPHEN IN MICE BRAIN

AUTHOR: MICHELE HINERASKY DA SILVA
ADVISOR: FELIX ALEXANDRE ANTUNES SOARES
Date and Place of the Defense: Santa Maria, 11th June 2012

Acetaminophen (APAP) is the analgesic most used in world, in therapeutic doses does not show toxicity, but in elevated doses can cause hepatic damage due the formation of his metabolic toxic N-acetyl-p-benzoquinonimine (NAPQI). The brain damage can occurs because hepatic damage a condition called hepatic encephalopathy, since the liver already show his function altered, like transform ammonia in urea, causing accumulation of ammonia in the brain, which is toxic for this organ. Furthermore, the NAPQI can cause oxidative damage and mitochondrial dysfunction on brain tissue. The diphenyl diselenide [$(PhSe)_2$] is an organoselenium compound that exhibit antioxidant activity and potential pharmacological. The aim of this study is investigated the ability of $(PhSe)_2$ in reversing the oxidative brain damage and mitochondrial dysfunction induced by a toxic dose of APAP. Mice received a APAP (600mg/kg), followed by a dose of $(PhSe)_2$ (15,6 mg/kg) 1 hour latter. Four hours after APAP administration, plasma was withdrawn from the mice and used for biochemical assays of aspastate aminotransferase (AST) and alanine aminotransferase (ALT) confirming the hepatic damage. The APAP administration increase lipid peroxidation, production of reactive oxygen species and decrease in activity of Na^+, K^+ - ATPase enzyme. Similary, APAP caused alteration on parameters of mitochondrial function. The treatment with $(PhSe)_2$ revert the cerebral damage induced by a single dose of APAP.

Keywords: oxidative damage, mitochondrial dysfunction, hepatic damage, antioxidant

LISTA DE ABREVIATURAS

$^{\bullet}NO$ – Óxido nítrico

ΔΨ – Potencial de membrana mitocondrial

ALT – Alanina aminotransferase

AINE – Anti-inflamatório não esteroidal

APAP – Paracetamol

AST – aspartato aminotransferase

BHE – Barreira hemato-encefálica

Ca^{+2} - Cálcio

CAT – Catalase

COX-2 – Cicloxigenase 2

CYP2E1- Citocromo P450 isoforma 2E1

DFCH – Diclorofluroisceína oxidada

EH – Encefalopatia hepática

ERO – Espécies reativas de oxigênio

GSH – Glutationa

GSH-Px - Glutationa Peroxidade

GST – Glutationa S transferase

MPT – Poro de transição mitocondrial

MTT - Metil tiazol tetrazólio

NAC – N-acetil-cisteína

Na^{+}, K^{+}- ATPase – Sódio, Potássio - ATPase

NAPQI – N-acetil-p-benzoquinoneimina

$ONOO^{-}$ - Peroxinitrito

$(PhSe)_2$ – Disseleneto de difenila

TBARS – Espécies reativas ao ácido tiobarbitírico

SOD - Superóxido dismutase

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INTRODUÇÃO

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APRESENTAÇÃO

No item **INTRODUÇÃO**, está descrita uma sucinta revisão bibliográfica sobre os temas trabalhados nesta dissertação.

O desenvolvimento referente a esta dissertação estão apresentados sob a forma de um artigo publicado na revista *Neurotoxicity Research* o qual se encontra alocado no item **ARTIGO CIENTÍFICO.** As seções Materiais e Métodos, Resultados, Discussão dos Resultados, Conclusão e Referências Bibliográficas, encontram-se no próprio artigo e representam a íntegra deste estudo.

Os itens **CONCLUSÃO e PERSPECTIVAS** são encontrados no final desta dissertação, apresentam interpretações e comentários gerais sobre a investigação desenvolvida.

As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente às citações que aparecem no item **INTRODUÇÃO** desta dissertação uma vez que o artigo científico contém as suas próprias referências.

1 INTRODUÇÃO

Paracetamol (*N*-acetil-*p*-aminofenol [APAP]) é atualmente o analgésico e antitérmico mais usado por humanos e animais (Dargan e Jones, 2002), sendo comercializado em formulações de medicamentos sozinho ou em combinação com outras substâncias (Sheen, Dillon *et al.*, 2002). Foi sintetizado por Morse em 1878, porém foi lançado no mercado somente em 1950 como substituinte da fenacetina, um analgésico muito utilizado na época que apresentava nefrotoxicidade como efeito colateral. O APAP está classificado como anti-inflamatório não esteroidal (AINE), apesar de não ter atividade anti-inflamatória e também não causar efeitos colaterais gastrointestinais como outros AINES (Bertolini, Ferrari *et al.*, 2006).

O efeito farmacológico do APAP é devido a inibição seletiva da enzima cicloxigenases-2 (COX-2) responsável pela síntese de prostaglandinas, mediadores da inflamação que causam dor e febre (Hinz, Cheremina *et al.*, 2008). Esse medicamento não apresenta toxicidade quando administrado em doses terapêuticas, porém uma quantidade diária acima de 4 gramas deve ser considerado tóxico, já que existem relatos de dano hepático em doses que variam entre 4 a 10 gramas (Fontana, 2008). O uso aumentado e de fácil acesso desse medicamento está sendo associado a diversos casos de tentativas de suicídio e de transplantes de fígado por cirrose hepática causada por intoxicação aguda e crônica com APAP (Larson, Polson *et al.*, 2005). Dados do *Center for Disease Control and Prevention* mostraram que o APAP representou 10% das causas de morte por suicídio entre os anos de 2005 e 2007 nos Estados Unidos, perdendo apenas para os antidepressivos.

Em doses terapêuticas, o APAP é metabolizado pelo fígado da seguinte maneira (Figura 1): em torno de 1 a 4% é excretado inalterado na urina, em torno de 90% sofre reações de glicuronização e sulfatação tornando-o inativo e hidrossolúvel para ser excretado na urina; de 5 a 15% é metabolizado pelas enzimas do citocromo P450 (CYP1A2, CYP3A1, CYP2A6 e principalmente, CYP2E1), as quais oxidam o APAP produzindo o seu intermediário reativo, o *N*-acetil-*p*-benzoquinoneimina (NAPQI) (Bertolini, Ferrari *et al.*, 2006). O NAPQI conjuga-se com a glutationa (GSH) hepática, inativando o intermediário reativo para então poder ser excretado também na urina (Mitchell, Jollow *et al.*, 1973).

Em uma situação de overdose de APAP as reações de glicuronização e sulfatação tornam-se saturadas, sobrecarregando a metabolização via CYP2E1,

formando assim uma grande quantidade de NAPQI. Esse excesso de NAPQI depleta a GSH disponível no fígado, deixando uma parte do metabólito ativo livre para ligar-se covalentemente às proteínas citosólicas e mitocondriais levando à hepatotoxicidade (Cohen, Hoivik *et al.*, 1998). A depleção de GSH contribui para o estresse oxidativo celular (Jaeschke, Knight *et al.*, 2003). Quando o NAPQI liga-se a alvos celulares críticos, como as proteínas mitocondriais, ocorre disfunção mitocondrial e inibição da síntese de ATP (Harman, Kyle *et al.*, 1991). Os hepatócitos sofrem subsequentemente falência energética, alteração na homeostase do cálcio, depleção de ATP, dano ao DNA e modificação de proteínas intracelulares (Andersson, Rundgren *et al.*, 1990). Estes eventos levam à morte celular fazendo com que esse órgão perca suas funções normais, incluindo a detoxificação da amônia ao transformá-la em uréia, além de afetar toda capacidade oxidante do organismo.

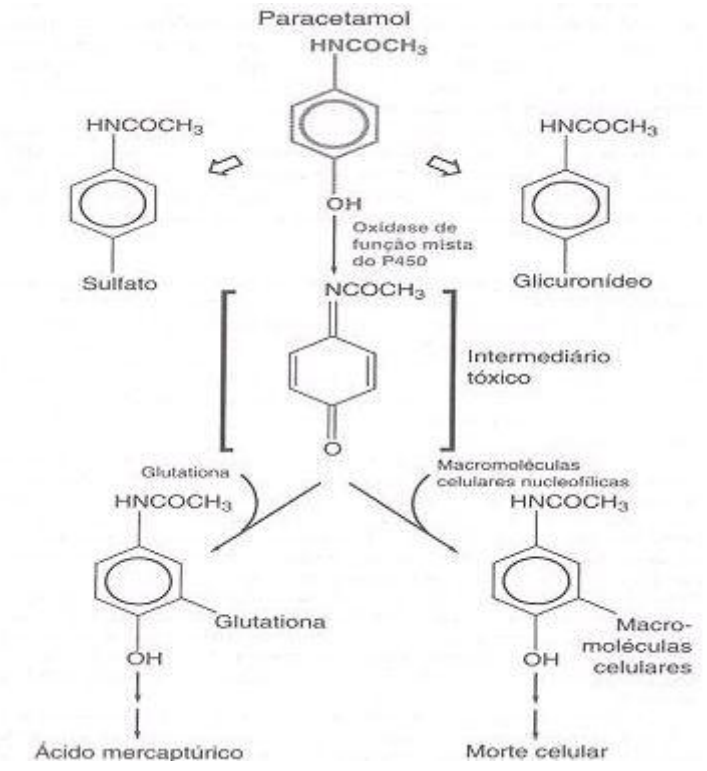

Figura 1 – Metabolização do APAP.

Um problema para a área médica consiste no tratamento de intoxicações com APAP, já que existe somente uma substância considerada antídoto, a N-acetil-cisteína (NAC) (Kozer e Koren, 2001). Um agente que apresenta efeitos

antioxidantes bem documentados frente a uma intoxicação por APAP (James, Mccullough *et al.*, 2003), por funcionar como uma precursora e substituinte de GSH (Buckpitt, Rollins *et al.*, 1979). Baseado em estudos clínicos, sabe-se que a eficácia da NAC é relacionada com sua administração o quanto antes possível após a overdose de APAP (Rumack e Matthew, 1975; Smilkstein, Douglas *et al.*, 1994). O uso da NAC também não mostra-se efetivo em todas as situações de overdose por APAP (Grewal e Racz, 1993), assim, estudos envolvendo novas estratégias de tratamento e também adjuvantes ao tratamento com NAC apresentam grande importância na intoxicação por APAP e outros agentes hepatotóxicos.

O fígado é o único órgão capaz de converter amônia em uréia através de reações do ciclo da ureia. Estando esse órgão com o seu funcionamento comprometido, devido ao dano causado por uma alta dose de APAP, ocorre o acúmulo de amônia no organismo. A amônia possui a capacidade de atravessar a barreira hemato-encefálica (BHE) atingindo o cérebro e desenvolvendo uma condição chamada de encefalopatia hepática (EH) (Diaz-Munoz e Tapia, 1989) (Felipo e Butterworth, 2002).

A EH é caracterizada como uma síndrome multifatorial em que o sistema nervoso central fica comprometido devido às consequências metabólicas de uma doença no fígado (Panatto, Jeremias *et al.*, 2011). Atualmente está descrito o envolvimento de alterações mitocondriais, estresse oxidativo (Rama Rao, Jayakumar *et al.*, 2003) (Ritter, Da Cunha *et al.*, 2006) e acúmulo de glutamina vinda da amônia (Takahashi, Koehler *et al.*, 1991; Hawkins, Jessy *et al.*, 1993) no mecanismo da EH. Além disso, o acúmulo de amônia no cérebro podem levar ao inchaço da mitocôndria e indução da abetura do porto de transição mitocondrial (MPT, do inglês *mitochondrial permeability transition*) (Zieminska, Dolinska *et al.*, 2000).

A mitocôndria tem um papel fundamental no funcionamento das células dependentes do metabolismo aeróbio, já que é a organela responsável pela produção de energia, através da síntese de ATP, pelo metabolismo intermediário e também por participar na morte celular, por ativar as vias de apoptose após a liberação do citocromo c. Essa organela é constituída por duas membranas, a membrana externa e a membrana interna, sendo a primeira menos seletiva que a outra, e entre ambas é determinado o espaço intermembrana. A membrana interna é formada por pregas que se expandem na matriz mitocondrial, denominadas de cristas mitocondriais. A maquinaria molecular para a produção energética é

constituída por enzimas que se encontram na matriz mitocondrial e por proteínas organizadas na membrana mitocondrial interna ou complexos proteicos que formam a chamada cadeia transportadora de elétrons.

O estresse oxidativo pode ser definido como um distúrbio entre o balanço pró-oxidante/antioxidante em favor da situação pró-oxidante, levando a um dano oxidativo que compromete a resposta redox celular. Esta situação pode ser resultado de uma diminuição dos sistemas antioxidantes ou aumento da produção de espécies reativas de oxigênio (ERO) e de nitrogênio (ERN) (Halliwell e Gutteridge, 2007). Os radicais livres são moléculas que possuem um elétron desemparelhado, tornando-se espécies eletrofílicas altamente reativas, que podem reagir com componentes celulares importantes, como proteínas, DNA e lipídeos (Timbrell, 2000). As ERO e ERN mais relevantes e deletérias originadas nos sistemas biológicos incluem: o radical ânion superóxido ($O_2^{\bullet}$), radical hidroxil ($^{\bullet}OH$), peróxido de hidrogênio (H_2O_2), óxido nítrico ($^{\bullet}NO$) e o peroxinitrito ($ONOO^-$) (Halliwell, 1999). Como mecanismo de proteção ao estresse oxidativo e aos danos promovidos por este processo no organismo, as células possuem sistemas de defesa antioxidantes, tais como as enzimas superóxido dismutase (SOD), glutationa peroxidase (GSH-Px), glutationa – S - transferase (GST), catalase (CAT). Distúrbios nestes sistemas de defesa podem levar ao acúmulo de ERO/ERN e acelerar o desenvolvimento de danos nas células.

Os estudos sobre a toxicidade das ERO têm sido acompanhados por pesquisas sobre o uso de antioxidantes, de moléculas com atividade neutralizante de espécies reativas e até mesmo de moléculas que estimulem a produção de antioxidantes endógenos. Esses estudos são importantes principalmente para o tratamento de doenças nas quais o estresse oxidativo está envolvido, sendo o motivo pelo qual tais estudos hoje estão em evidência na comunidade científica (Halliwell, 2001) (Dodd, Dean *et al.*, 2008) (Brambilla, Mancuso *et al.*, 2008).

O tecido cerebral é altamente suscetível aos efeitos do estresse oxidativo devido ao seu alto consumo de oxigênio e, ainda, por possuir poucas defesas antioxidantes, elevadas concentrações de ferro livre e altas concentrações de ácidos graxos insaturados, o que propicia a peroxidação lipídica (Floyd, 1999). Estudo prévio mostrou que ocorre uma diminuição de GSH após uma overdose de APAP. Sabe-se que a GSH apresenta um importante papel nas funções celulares e assim, manter sua homeostase é essencial ao organismo. Diminuição nos níveis de GSH

torna o tecido cerebral mais suscetível à ação do APAP e, consequentemente, do seu metabólito ativo (Bajt, Knight *et al.*, 2004). O APAP possui a capacidade de permear a BHE (Courad, Besse *et al.*, 2001) e atingir diretamente o cérebro, estudos demonstraram que a principal isoforma do CYP450 que metaboliza o APAP, o CYP2E1, também está presente no cérebro (Hansson, Tindberg *et al.*, 1990; Howard, Miksys *et al.*, 2003), indicando que ocorre a formação do NAPQI diretamente nesse órgão. Assim, acredita-se que o NAPQI esteja depletando diretamente a GSH cerebral e no caso de uma overdose, o metabólito tóxico causa estresse oxidativo diretamente no cérebro, além dos danos causados pelo acúmulo de amônia vinda do dano hepático.

O estresse oxidativo pode afetar diversas estruturas e componentes funcionais da mitocôndria, incluindo o DNA mitocondrial, proteínas e membranas. Isto pode resultar em peroxidação lipídica e ligações danosas com proteínas, com mudanças simultâneas na taxa de respiração, síntese de ATP, fluidez e permeabilidade da membrana e rompimento na homeostase do Ca^{2+}, tudo isso pode causar ativação da via da apoptose (Acuna, Escames *et al.*, 2002). Essa organela é alvo de ligação covalente entre suas proteínas e o NAPQI (Tirmenstein e Nelson, 1989; Pumford, Hinson *et al.*, 1990; Gupta, Rogers *et al.*, 1997; Pumford, Halmes *et al.*, 1997), esse fator pode ser um dos principais responsáveis pelas mudanças estruturais vistas na célula (Placke, Ginsberg *et al.*, 1987) e também pela inibição da respiração mitocondrial (Meyers, Beierschmitt *et al.*, 1988) após uma overdose de APAP. Isto levará a disfunção mitocondrial que por sua vez desencadeará em mais estresse oxidativo na mitocôndria (Jaeschke, 1990; Tirmenstein e Nelson, 1990). Esses fatores causarão um aumento na permeabilidade da membrana mitocondrial e consequentemente ruptura da mesma, caracterizando a abertura do MPT.

O MPT consiste em um canal para ânion voltagem dependente na membrana mitocondrial externa, translocador de nucleotídeo de adenina na membrana mitocondrial interna e ciclofilina D na matriz (Figura 2) (Kim, He *et al.*, 2003). O MPT está envolvido na patogênese do dano hepático induzido por APAP (Jaeschke, Knight *et al.*, 2003). Diferentes agentes e condições podem iniciar a abertura do MPT, incluindo o Ca^{2+}, fosfato inorgânico, pH alcalino e as EROS (Gunter e Pfeiffer, 1990). Acredita-se que o estresse oxidativo causado pelo NAPQI na mitocôndria seja o responsável pela indução da abertura do MPT (Masubuchi, Suda *et al.*, 2005). Além disso, o NAPQI tem a capacidade de oxidar o NADPH e os grupamentos tióis

das proteínas da mitocôndria (Weis, Moore *et al.*, 1990), se os grupamentos tióis das proteínas são oxidados, consequentemente ocorrerá ligação cruzada entre essas proteínas, mudando a conformação proteica e desencadeando a abertura do MPT (Masubuchi, Suda *et al.*, 2005).

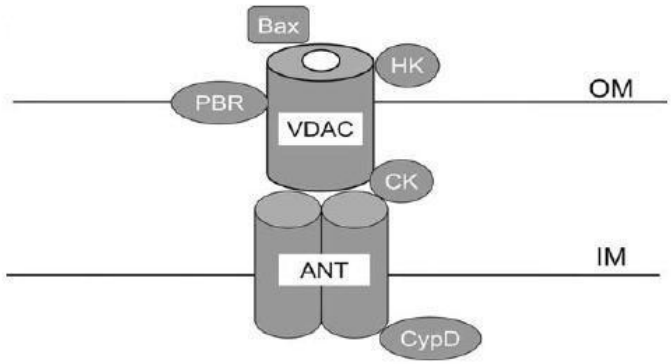

Figura 2 – Poro de transição mitocondrial. OM (membrana mitocondrial externa); IM (membrana mitocondrial interna); VDAC (canal para ânion voltagem dependente); ANT (translocador de nucleotídeo de adenina); CypD (ciclofilina D); PBR (receptor benzodiazepínico periférico); HK (hexoquinase); CK (creatina quinase).

A abertura do MPT compromete a integridade da membrana mitocondrial interna, o que resulta em duas consequências principais (Armstrong, 2006). Primeiro, a membrana mitocondrial interna torna-se permeável à saída de prótons, dispersando o gradiente dos mesmos e desacoplando a fosforilação oxidativa. Assim, a enzima responsável pela síntese de ATP, a F_1F_o-ATPase torna-se responsável por hidrolisar ATP, com o objetivo de manter o potencial de membrana mitocondrial ($\Delta\Psi$), e a concentração intracelular de ATP diminui. A homeostase metabólica fica comprometida devido a ativação de fosfolipases, proteases e nucleases o que induz a morte celular por necrose. E como segunda consequência, a perda de seletividade da membrana mitocondrial interna deixa que a água passe livremente por ela, resultando em inchaço da matriz mitocondrial e ruptura mecânica da membrana mitocondrial externa. A ruptura da membrana mitocondrial externa libera proteínas pró-apoptóticas, como por exemplo o citocromo c, uma proteína que pertence ao espaço intermembranas e quando liberado para o citosol da células ativa a cascata da apoptose. A relação da formação MPT também está bem estabelecida com a indução da morte celular (Armstrong, 2006).

Esses estudos realizados relacionando a abertura do MPT com a patogênese de uma overdose por APAP foram realizados em mitocôndrias hepáticas. Por não existirem estudos que mostrem como o APAP afeta o tecido cerebral, acredita-se que o APAP é metabolizado diretamente no cérebro, formando NAPQI e gerando estresse oxidativo diretamente nesse órgão. Assim, o mecanismo pelo qual ocorra dano cerebral seja semelhante ao que ocorre no tecido hepático, uma vez que o cérebro apresenta grande quantidade de mitocôndrias e que a disfunção mitocondrial já está relacionada com a patogênese de várias doenças cerebrais (Beal, 1992; Blass, 2001; 2002; Schurr, 2002; Land, Morgan-Hughes *et al.*, 2004; Beal, 2005; Lin e Beal, 2006).

O Selênio é um calcogênio do grupo 16 da tabela periódica descoberto em 1817, podendo apresentar-se sob quatro estados químicos de oxidação: selenato (Se^{+6}), selenito (Se^{+4}), selênio elementar (Se^{0}) e seleneto (Se^{-2}). Compartilha propriedades químicas e físicas com o enxofre. Essa similaridade permite que o selênio substitua ao enxofre, promovendo interações selênio-enxofre nos sistemas biológicos (Stadtman, 1980). O selênio é um elemento traço essencial, cuja essencialidade nutricional foi demonstrada em 1957, em ratos (Schwartz e Foltz, 1957). Atualmente, sabe-se que baixos níveis de selênio podem levar à predisposição para o desenvolvimento de algumas doenças, tais como câncer, esclerose, doença cardiovascular, cirrose e diabetes (Navarro-Alarcon e Lopez-Martinez, 2000). Este calcogênio apresenta um grande número de funções biológicas, sendo a mais importante a de antioxidante, descoberta através de sua participação no sítio ativo da enzima GSH-Px, em 1973 (Rotruck, Pope *et al.*, 1973). Sabe-se que as moléculas contendo selênio, como por exemplo o disseleneto de difenila $(PhSe)_2$, podem ser melhores nucleófilos do que os antioxidantes clássicos (Arteel e Sies, 2001).

A partir da década de 30, os organocalcogênios tem sido alvo de interesse para químicos orgânicos em virtude da descoberta de aplicações sintéticas (Comasseto, 1983), sendo importantes intermediários e reagentes muito utilizados na síntese orgânica (Paulmier, 1986) (Braga, et. al., 1996).

O interesse em pesquisas envolvendo compostos orgânicos de selênio tem-se intensificado devido às suas diversas propriedades (Nogueira, Zeni *et al.*, 2004), em particular o $(PhSe)_2$, um composto altamente lipofílico e que apresenta diversas propriedades farmacológicas. Este composto apresenta papel protetor em uma

variedade de modelos experimentais associados à produção exacerbada de radicais livres (Rossato, Ketzer *et al.*, 2002; Ghisleni, Porciuncula *et al.*, 2003; Burger, Fachinetto *et al.*, 2004; Meotti, Stangherlin *et al.*, 2004; Borges, Rocha *et al.*, 2005). Em modelo experimental de diabetes, o tratamento com $(PhSe)_2$ reduziu significativamente a hiperglicemia e a indução de estresse oxidativo em diferentes tecidos de roedores (Barbosa, Rocha *et al.*, 2006; Barbosa, Rocha *et al.*, 2008). Adicionalmente, este composto foi capaz atuar beneficamente em modelos experimentais de inflamação e dor (Nogueira, Quinhones *et al.*, 2003; Savegnago, Trevisan *et al.*, 2006; Savegnago, Pinto *et al.*, 2007; Savegnago, Jesse *et al.*, 2008), e na indução de hepatotoxicidade em roedores (Borges, Borges *et al.*, 2005; Borges, Brandao *et al.*, 2008; Wilhelm, Jesse *et al.*, 2009). O $(PhSe)_2$ também demonstrou proteger as plaquetas humanas contra peroxidação lipídica induzida pelo nitroprussiato de sódio, um doador de óxido nítrico, e reativou a atividade da GSH-Px nestas células (Posser, Moretto *et al.*, 2006). Além de apresentar efeito anti-ulcerogênico (Savegnago, Trevisan *et al.*, 2006; Ineu, Pereira *et al.*, 2008) e neuroprotetor (Ghisleni, Porciuncula *et al.*, 2003). Outra característica importante desse composto é que ele mostrou-se menos tóxico que o ebselen (Meotti, Stangherlin *et al.*, 2004). Entretanto, não existem estudos na literatura mostrando o potencial protetor do $(PhSe)_2$ em mitocôndrias cerebrais, apenas um estudo relacionando a toxicidade do $(PhSe)_2$ em mitocôndrias, porém é um trabalho realizado *in vitro* e em doses maiores que as usadas nesse estudo (Puntel, Roos *et al.*, 2010).

Tendo em vista o grande número de casos de intoxicação por APAP e o fato de não existirem terapias alternativas e efetivas para o uso da NAC, este estudo tem por objetivo reverter o dano oxidativo cerebral e mitocondrial induzido por um dose hepatotóxica de paracetamol utilizando o composto de selênio $(PhSe)_2$.

2 OBJETIVOS

2.1 Objetivo geral

Avaliar a possível reversão do estresse oxidativo e do dano mitocondrial cerebral causado por uma dose tóxica de paracetamol em camundongos, através da utilização do disseleneto de difenila.

2.2 Objetivos específicos

- Avaliar o efeito do $(PhSe)_2$ sobre os marcadores de estresse oxidativo no tecido cerebral.
- Avaliar o efeito do $(PhSe)_2$ sobre a atividade de enzimas antioxidantes no tecido cerebral.
- Avaliar o efeito do $(PhSe)_2$ sobre a viabilidade celular do tecido cerebral.
- Avaliar os parâmetros de funcionalidade mitocondrial: potencial de membrana mitocondrial, inchaço mitocondrial e produção de espécies reativas de oxigênio.

3 DESENVOLVIMENTO

O desenvolvimento que faz parte desta dissertação está apresentado sob a forma de artigo científico. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se no próprio artigo. O artigo encontra-se na formatação de publicação da revista científica *Neurotoxicity Research.*

ARTIGO: DANO AGUDO CEREBRAL INDUZIDO POR PARACETAMOL EM CAMUNDONGOS: EFEITOS DO DISSELENETO DE DIFENILA SOBRE O ESTRESSE OXIDATIVO E DISFUNÇÃO MITOCONDRIAL

Artigo científico publicado na revista *Neurotoxicity Research*

ACUTE BRAIN DAMAGE INDUCED BY ACETAMINOPHEN IN MICE: EFFECT OF DIPHENYL DISELENIDE ON OXIDATIVE STRESS AND MITOCHONDRIAL DYSFUNCTION

Michele Hinerasky da Silva, Edovando José Flores da Rosa, Nélson Rodrigues de Carvalho, Fernando Dobrachinski, João Batista Teixeira da Rocha, Jose L. Mauriz, Javier González-Gallego, Félix Alexandre Antunes Soares

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Acute Brain Damage Induced by Acetaminophen in Mice: Effect of Diphenyl Diselenide on Oxidative Stress and Mitochondrial Dysfunction

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Abstract Organoselenium compounds exhibit antioxidant activity, as well as a variety of biological activities, with potential pharmacological and therapeutic applications. The aim of this study was to investigate the effect of diphenyl diselenide $(PhSe)_2$ in reversing oxidative brain damage and mitochondrial dysfunction caused by administration of acetaminophen (APAP) in mice. Mice received a toxic dose of APAP, followed by a dose of $(PhSe)_2$ 1 h later. Four hours after the administration of APAP, plasma was withdrawn from the mice and used for biochemical assays of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as markers of hepatotoxicity. Brain homogenate was examined to determine oxidative stress. Isolated brain mitochondria were examined to quantify mitochondrial transmembrane's electrical potential and mitochondrial swelling and to estimate reactive oxygen species (ROS) production. APAP administration caused an increase in plasma ALT and AST activities. APAP administration also caused a significant increase in the levels of thiobarbituric acid reactive substances (TBARS) and dichlorofluorescein oxidation in brain homogenate. Similarly, mitochondrial swelling and ROS production increased after APAP administration. APAP treatment also caused a decrease in Na^+ , K^+ - ATPase activity and in mitochondrial membrane potential. These alterations observed in the brain of APAP-treated mice were restored by $(PhSe)_2$. Glutathione levels were decreased by APAP, but $(PhSe)_2$ did not reverse this change. Treatment with $(PhSe)_2$ after APAP administration can reverse the neurotoxicity caused by a single toxic dose of APAP. The neuroprotective effect of $(PhSe)_2$ is likely associated with its antioxidant properties.

Keywords Brain · Oxidative stress · Mitochondrial dysfunction · Organoselenium compounds · Acetaminophen · Liver damage

Introduction

Acetaminophen [*N*-acetyl-*p*-aminophenol (APAP)] is an analgesic and antipyretic agent that is widely used in humans and animals (Dargan and Jones 2002). APAP is used alone and in combination with other drugs for its analgesic and antipyretic properties (Sheen et al. 2002). An overdose of APAP (suicidal or accidental) can cause acute liver failure.

Under normal conditions, APAP is metabolized into the reactive intermediate *N*-acetyl-*p*-benzoquinonimine (NAPQI), which is normally detoxified by glutathione (GSH). In an APAP overdose, GSH is depleted by the NAPQI detoxification process, and the excess NAPQI causes oxidative stress (Cohen et al. 1998; Bessems and Vermeulen 2001), which leads to a disruption in normal liver functions, one of which is to detoxify ammonia. The accumulation of ammonia can be very toxic to the brain,

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leading to the development of a condition called hepatic encephalopathy (Diaz-Munoz and Tapia 1989). Moreover, the formation of reactive oxygen species (ROS) from the liver affects the antioxidant capacity of the whole organism. These processes are particularly destructive for the brain, since brain tissue is not highly enriched in antioxidant protective defenses (Floyd 1999).

Brain tissue is highly susceptible to oxidative stress owing to its high content of unsaturated fatty acids, high oxygen consumption, and poorly developed oxidative defense mechanisms (Floyd 1999). A previous study revealed a significant decrease in cerebral GSH levels after an APAP overdose in rats. GSH plays an important role in cellular functions, and the maintenance of GSH homeostasis is essential for the organism. GSH reduction makes brain tissue more susceptible to the action of APAP and its toxic metabolites.

Oxidative damage can affect several structural and functional components of mitochondria, including mitochondrial DNA (mtDNA), proteins and membranes. This can result in progressive lipid peroxidation and crosslinking damage in proteins, with simultaneous changes in the respiration rate, ATP synthesis, membrane fluidity and permeability, and Ca^{2+} homeostasis, all of which activate apoptosis (Acuna et al. 2002). Since Hunter et al. (1976) first characterized the mitochondrial permeability transition (MPT), its formation has been implicated as a key mechanism underlying cell death (Armstrong 2006). The opening of high-conductance permeability transition (PT) pores in the mitochondrial inner membrane initiates the MPT. Many agents and conditions are promoters of MPT, including Ca^{2+} , inorganic phosphate, an alkaline pH, and ROS (Gunter and Pfeiffer 1990). MPT leads to mitochondrial depolarization, uncoupling of oxidative phosphorylation, and large amplitude swelling, which, in turn, can lead to ATP depletion and cell death (Jaeschke 1990).

In the last decade, the interest in selenium compounds has intensified, because a variety of these compounds have pharmacological properties (Nogueira et al. 2004). In particular, diphenyl diselenide $(PhSe)_2$, the simplest of the diaryl diselenides and a lipophilic organic compound of selenium, has numerous pharmacological properties, including antiulcerogenic (Savegnago et al. 2006; Ineu et al. 2008), anti-inflammatory (Savegnago et al. 2007), neuroprotective (Ghisleni et al. 2003), and hepatoprotective (Wilhelm et al. 2009) activities. Moreover, $(PhSe)_2$ has antioxidant properties in a variety of in vitro and in vivo models of oxidative stress (Rossato et al. 2002; Prigol et al. 2009; Luchese et al. 2009). Of particular therapeutic significance, $(PhSe)_2$ exhibits improved antioxidant effects and fewer toxic properties in rodents than ebselen (Meotti et al. 2004), which has been used with borderline efficacy in clinical trials (Saito et al. 1998). However, to the best of our knowledge, data demonstrating the potential protective effect of $(PhSe)_2$ on cerebral mitochondria are lacking in the literature. There is one study (Puntel et al. 2010) showing the toxicity of $(PhSe)_2$ on liver mitochondria, but this study was conducted ex vivo and in doses different than those that we used in this study.

Therefore, we investigated if $(PhSe)_2$ could reverse the oxidative damage and mitochondrial dysfunction in the brains of mice caused by a neurotoxic dose of APAP.

Materials and Methods

Chemicals

$(PhSe)_2$ was prepared according to the literature method according to Paulmier (1986) and was dissolved in canola oil. Analysis of the 1H NMR and 13C NMR spectra showed that $(PhSe)_2$ presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of $(PhSe)_2$ (99.9%) was determined by GC/HPLC.

Thiobarbituric acid (TBA), 2′-7′-dichlorofluorescein (DCF), trichloroacetic acid (TCA) and nucleotides were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Animals

Male swiss albino mice were from our breeding colony, and weighing between 25 and 35 g. The animals were kept in a separate animal room, on a 12-h light/dark cycle, at a room temperature of 22 ± 2°C and with free access to food and water. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources of the Federal University of Santa Maria, Brazil.

Experimental Procedure

The animals were randomly divided into four groups: (I) control; (II) $(PhSe)_2$; (III) APAP; and (IV) APAP + $(PhSe)_2$. Mice of groups III and IV were injected intraperitoneally (i.p.) with a single dose of APAP (600 mg/kg body weight dissolved in warm buffered saline, 20 ml/kg (Chan et al. 2001). One hour later, animals of groups II and IV were injected i.p. with $(PhSe)_2$ (15.6 mg/kg, i.p. dissolved in canola oil, 2.5 ml/kg) (Rosa et al. 2007). Animals of group I received only vehicle i.p. (saline and canola oil).

Four hours after administration of APAP or saline, mice were euthanized and the brain of animals was removed, dissected. The samples of brain were homogenized in 50 mM Tris–HCl, pH 7.4 (1/10, w/v), centrifuged at

3,000×*g* for 10 min to yield the low-speed supernatant fractions that were used for different biochemical assays in all trials. Blood was collected in order to perform AST/ALT analysis.

Mitochondrial Isolation

Brain mitochondria were isolated as previously described by Bhattacharya et al. (1991), with some modifications. The brain was rapidly removed and immersed in ice-cold isolation buffer I (100 mM sucrose, 10 mM EDTA, 100 mM Tris–HCl, 46 mM KCl, at pH 7.4). The tissue was then homogenized, and the resulting suspension was centrifuged for 3 min at 2,000×*g* in a Hitachi CR21E centrifuge. After centrifugation, the supernatant was recentrifuged for 10 min at 12,000×*g*. The pellet was resuspended in isolation buffer II (100 mM sucrose, 10 mM EDTA, 100 mM Tris–HCl, 46 mM KCl, and 0.5% bovine serum albumin (BSA) free of fatty acids, at pH 7.4) and recentrifuged at 12,000×*g* for 10 min. The supernatant was decanted, and the final pellet was gently washed and resuspended in 125 µl of isolation buffer III (270 mM mannitol, 70 mM sucrose, 0.02 mM EDTA, 20 mM Tris–HCl, 1 mM K2HPO4, at pH 7.4).

ALT and AST Serum Determination

Plasma enzymes aspartate (AST) and alanine aminotransferases (ALT) activities were used as biochemical markers for the early acute hepatic damage. Transaminases were determined by the colorimetric method of Reitman and Frankel (1957).

Thiobarbituric Acid Reactive Substance (TBARS) Level Determination

Lipid peroxidation was estimated by measuring TBARS and expressed in terms of malondialdehyde (MDA) content, according to the method of Ohkawa et al. (1979). In this method, MDA, an end product of fatty acid peroxidation, reacts with TBA to form a colored complex. In Brief, the supernatant fraction of brain was incubated at 100°C for 60 min in acid medium containing 8.1% sodium dodecyl sulfate, 0.5 ml of acetic acid buffer (500 mM, pH 3.4) and 0.6% TBA. TBARS levels were measured at 532 nm, and the absorbance was compared with the standard curve using malondialdehyde.

Oxidized Diclorofluoresceine (DCFH) Levels Determination in Brain Homogenate

2′-7′-Dichlorofluorescein (DCFH) levels were determined as an index of the peroxide production by the cellular components. This experimental method of analysis is based on the deacetylation of the probe DCFH-DA, and its subsequent oxidation by reactive species to DCFH, a highly fluorescent compound (Halliwell and Gutteridge 2007). The supernatant fractions of brain homogenate were added to a medium containing Tris–HCl buffer (10 mM; pH 7.4) and DCFH-DA (1 mM). After DCFH-DA addition, the medium was incubated in the dark for 1 h until the start of fluorescence measurement procedure (excitation at 488 nm and emission at 525 nm, and both slit widths used were at 1.5 nm). DCFH oxidation was determined using a standard curve of DCF and results were corrected by the protein content.

Superoxide Dismutase (SOD) Activity

The SOD enzyme activity was determined in brain according to the method proposed by Misra and Fridovich (1972). This method is based on the capacity of SOD in inhibiting autoxidation of adrenaline to adrenochrome. In Brief, the supernatant fraction (100 µl) was added to a medium containing sodium bicarbonate–carbonate buffer (50 mM; pH 10.2) and adrenaline (0.4 mM). The kinetic analysis of SOD was started after adrenaline addition, and the color reaction was measured at 480 nm.

Sodium Potassium (Na^+ , K^+ -ATPase) Activity

The cerebral (Na^+ , K^+ -ATPase) activity was determined according to the method proposed by Musbeck (1997) with some modifications. In Brief, 20 µl supernatant fraction of brain homogenate was added to a reaction medium containing NaCl (125 mM), $MgCl_2$ (3.0 mM), KCl (20 mM), and Tris–HCl buffer (50 mM, pH 7.4), with or without ouabain (5 mM). The method for ATPase activity measurement was based on the determination of the inorganic phosphate (Pi) released to the reaction medium by the hydrolysis of the ATP according to the method proposed by Atkinson et al. (1973). The reaction was initiated with the addition of the substrate ATP (1.5 mM) to the reaction medium, and was finished by the addition of the color reagent (1 ml) containing ammonium molybdate (2%) triton-100X (5%), and H_2SO_4 1.8 M (10%) after 15 min of incubation at 37°C. The formed molybdate–Pi complexes were measured spectrophotometrically at 405 nm. The values were calculated in relation to a standard curve constructed with Pi at known concentration sand also corrected by the protein content.

Methyl-Tetrazolium (MTT) Reduction Levels

MTT reduction levels were determined as an index of the dehydrogenase enzymes functions, which are involved in

the cellular viability (Bernas and Dobrucki 2002). 100 µl of supernatant fraction of brain homogenate was added to a medium containing 0.5 mg/ml of MTT and were incubated in the dark for 1 h at 37°C. The MTT reduction reaction was stopped by the addition of 1 ml of dimethylsulfoxide (DMSO). The formed formazan levels were determined spectrophotometrically at 570 nm and the results were corrected by the protein content (Mosmann 1983).

GSH Levels

The level of total GSH was measured by the fluorimetric method of Hissin and Hilf (1976). This method is based on the principle that GSH reacts specifically with opthaldehyde (OPT) at pH 8 resulting in the formation of a highly fluorescent product which is activated at 350 nm with an emission at 420 nm. Results were expressed as nmol of GSH/mg protein.

Measurements of Mitochondrial Membrane Potential ($\Delta\Psi$)

Mitochondrial was estimated by fluorescence changes in safranine (3 µM) recorded using RF-5301 Shimadzu spectrofluorometer (Kyoto, Japan) operating at excitation and emission wavelengths of 495 and 535 nm, respectively with slit widths of 1.5 nm (Guo et al. 1998), using samples of 0.5 mg/ml of protein. $\Delta\Psi$ is presented as arbitrary fluorescence units per second (AFU/s).

Mitochondrial Swelling

Measurement of mitochondrial swelling was performed in a RF-5301 Shimadzu spectrofluorometer at 600 nm [slit 1.5 nm for excitation and emission (Votyakova and Reynolds 2005)] using samples of 0.12 mg/ml of protein. Data for mitochondrial swelling are expressed as arbitrary absorbance units per second (AAU/s).

Estimation of ROS Production in Mitochondria

The mitochondrial generation of ROS was determined spectrofluorimetrically, using the membrane permeable fluorescent dye H2-DCFDA (Garcia-Ruiz et al. 1997) (1 µM). Fluorescence was determined at 488 nm for excitation and 525 nm for emission, with slit widths of 3 nm, using buffer III as incubation medium.

Protein Determination

The protein content was determined according to Bradford (1976) using bovine serum albumin (BSA) as standard.

Statistical Analysis

Statistical analysis was performed using GraphPad (version 5.0 for Macintosh OSX, GraphPad Software, San Diego, CA). Significance was assessed by one-way analysis of variance (ANOVA), followed by Newman–Keuls's Test for post-hoc comparison. Values of $P < 0.05$ were considered statistically significant.

Results

ALT and AST Activities

Acute exposure to 600 mg/kg of APAP caused a significant increase ($P < 0.05$) in ALT and AST activities compared with the control group (Fig. 1). Treatment with $(PhSe)_2$

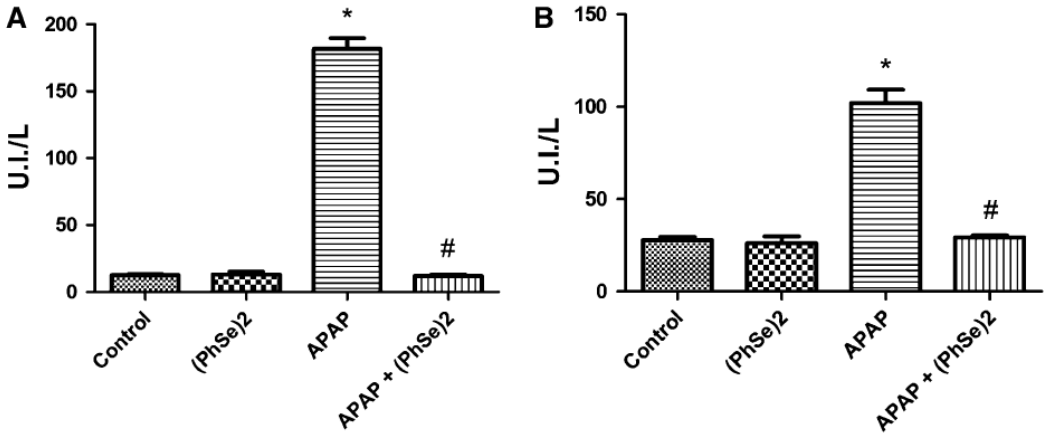


Fig. 1 Effects of treatment with APAP and $(PhSe)_2$ on the ALT (**a**) and AST (**b**) activity in serum of mice. Data are expressed as means ± SEM, ($n = 4$–5). ALT/ AST activities are expressed as U.I./L. * Denotes $P < 0.05$ as compared with the control group. # Denotes $P < 0.05$ as compared with the APAP group

after administration of APAP caused a significant decrease in ALT (Fig. 1a) and AST (Fig. 1b) activities compared with the APAP group.

Cerebral Thiobarbituric Acid Reactive Substance (TBARS) Levels

APAP administration caused a significant increase in TBARS compared with the control group (Fig. 2; $P < 0.05$). Moreover, statistical analysis demonstrated that the group treated with APAP followed by $(PhSe)_2$ had a significant decrease in TBARS levels in brain compared with the APAP group (Fig. 2; $P < 0.05$).

Diclorofluoresceine (DCFH) Oxidation Levels in Brain Homogenate

APAP treatment caused a significant increase in DCFH oxidation in brain homogenates compared with the control group (Fig. 3; $P < 0.05$). $(PhSe)_2$ treatment partially blunted the increase in DCFH oxidation caused by APAP exposure, but the oxidation levels were not significantly different from the APAP or control groups.

Superoxide Dismutase (SOD) Activity

APAP caused a significant increase ($P < 0.05$) in the SOD activity compared to the control group. $(PhSe)_2$ treatment blunted the stimulation of SOD activity caused by APAP (Fig. 4; $P < 0.05$).

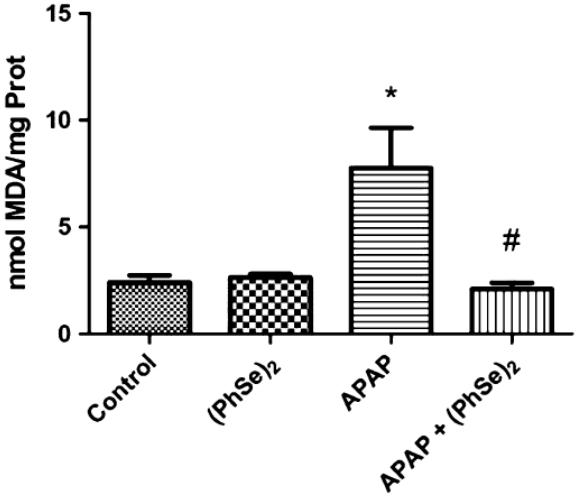


Fig. 2 Effects of treatment with APAP and $(PhSe)_2$ on TBARS in brain of mice. Data are expressed as means ± SEM, ($n = 4$–5). TBARS levels are expressed as nmol of MDA/mg protein. * Denotes $P < 0.05$ as compared with the control group. # Denotes $P < 0.05$ as compared with the APAP group

Na^+ , K^+ -ATPase Activity

As shown in Fig. 5, there was a significant reduction ($P < 0.05$) in the Na^+ , K^+ -ATPase activity in the brain of APAP-treated animals compared with the control group. In the group treated with APAP and $(PhSe)_2$, the Na^+ , K^+ -ATPase activity was higher than those in control and APAP groups (Fig. 5; $P < 0.05$).

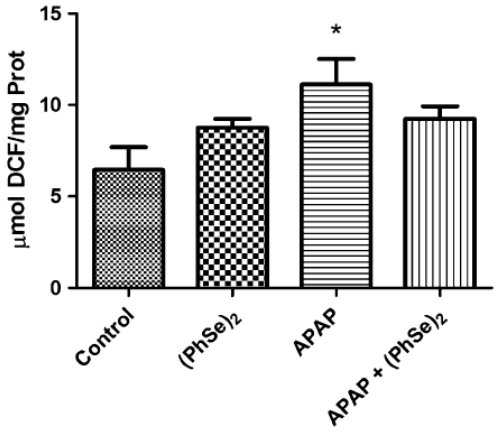


Fig. 3 Effects of treatment with APAP and $(PhSe)_2$ on reactive species (ROS) production (DCFH–DA) in brain of mice. Data are expressed as means ± SEM, ($n = 4$–5). Data of ROS levels are presented as µmol of DCF/mg protein. * Denotes $P < 0.05$ as compared with the control group

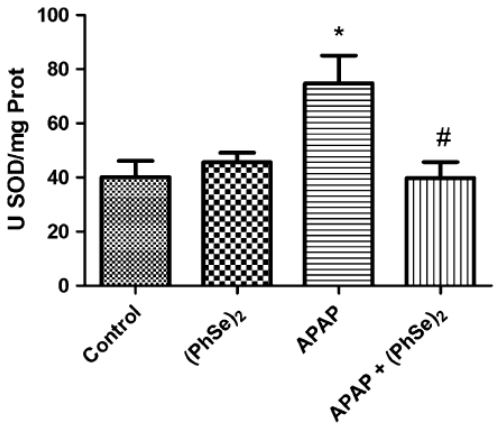


Fig. 4 Effects of treatment with APAP and $(PhSe)_2$ on on the activity of superoxide dismutase (SOD) in brain of mice. Data are expressed as means ± SEM, ($n = 4$–5). SOD activities are expressed as U.I. SOD/mg protein. * Denotes $P < 0.05$ as compared with the control group. # Denotes $P < 0.05$ as compared with the APAP group

MTT Reduction Levels

The APAP group exhibited a significant decrease in brain cell viability compared with the control group (Fig. 6; $P < 0.05$). $(PhSe)_2$ treatment blunted this decrease in cell viability caused by APAP compared with the control group.

GSH Levels

APAP administration caused a significant reduction in brain GSH levels compared with the control group, which was not modified by treatment with $(PhSe)_2$ (Fig. 7; $P < 0.05$).

Mitochondrial Membrane Potential Determination ($\Delta\Psi$)

Acute exposure to APAP caused a significant decrease in mitochondrial membrane potential compared with the control group, $(PhSe)_2$ treatment after APAP exposure returned the mitochondrial membrane potential that of to control levels (Fig. 8; $P < 0.05$).

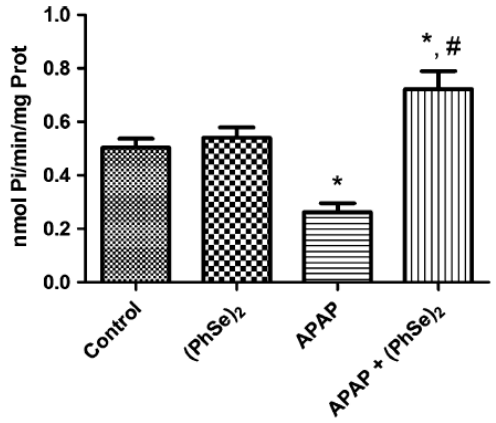


Fig. 5 Effects of treatment with APAP and $(PhSe)_2$ on the activity of the Na^+, K^+ - ATPase in brain of mice. Data are expressed as means ± SEM, ($n = 4$–5). Na^+, K^+ -ATPase activities are expressed as nmol Pi/min/mg protein. * Denotes $P < 0.05$ as compared with the control group. # Denotes $P < 0.05$ as compared with the APAP group

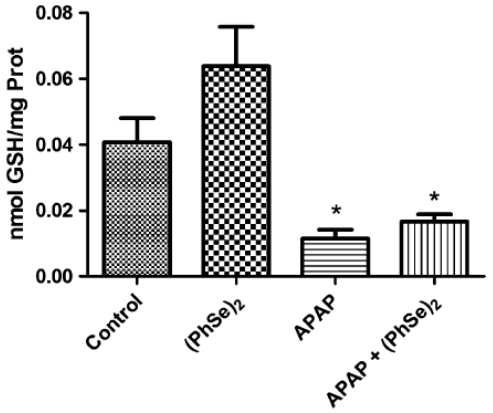


Fig. 7 Effects of treatment with APAP and $(PhSe)_2$ on the GSH levels in brain of mice. Data are expressed as means ± SEM, ($n = 4$–5). GSH level are expressed as nmol GSH/mg protein. * Denotes $P < 0.05$ as compared with the control group

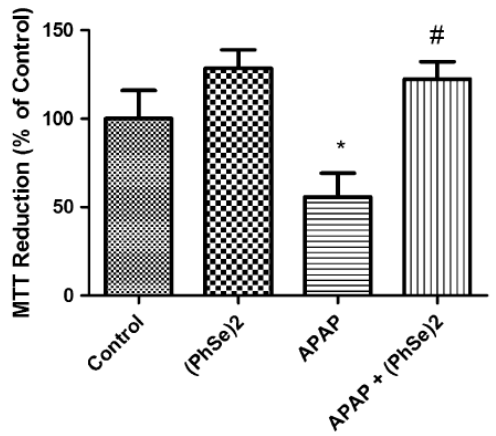


Fig. 6 Effects of treatment with APAP and $(PhSe)_2$ on cell viability in brain of mice. Data are expressed as means ± SEM, ($n = 4$–5). MTT reduction are expressed as percentage of control. * Denotes $P < 0.05$ as compared with the control group. # Denotes $P < 0.05$ as compared with the APAP group

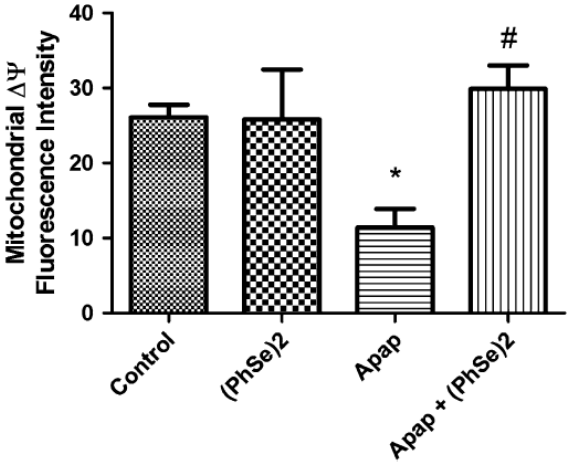


Fig. 8 Effects of treatment with APAP and $(PhSe)_2$ on $\Delta\Psi$ in isolated mice brain mitochondria. Data are expressed as means ± SEM, ($n = 5$). Results are expressed as arbitrary fluorescence intensity (A.F.I.). * Denotes $P < 0.05$ as compared with the control group. # Denotes $P < 0.05$ as compared with the APAP group

Mitochondrial Swelling

Acute exposure to APAP caused a significant increase in mitochondrial swelling compared with the control group (Fig. 9; $P < 0.05$). Moreover, $(PhSe)_2$ treatment was effective in reducing the mitochondrial swelling to control levels ($P < 0.05$).

Production of ROS in Mitochondria

APAP administration induced a significant increase in DFCH oxidation compared with the control group. This effect was significantly reduced by $(PhSe)_2$ treatment (Fig. 10; $P < 0.05$).

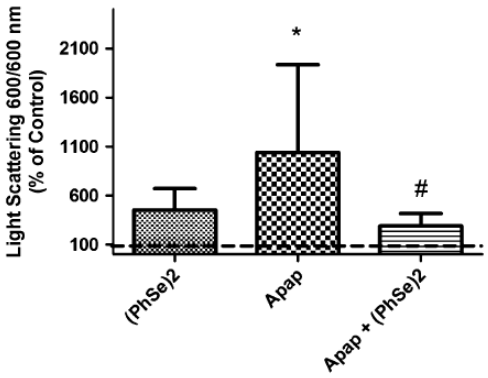


Fig. 9 Effects of treatment with APAP and $(PhSe)_2$ on mitochondrial swelling in isolated mice brain mitochondria. Data are expressed as means ± SEM, ($n = 5$). Results are expressed as percentage of control.* Denotes $P < 0.05$ as compared with the control group. # Denotes $P < 0.05$ as compared with the APAP group

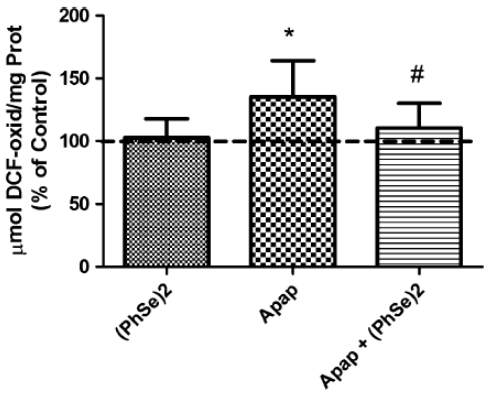


Fig. 10 Effects of treatment with APAP and $(PhSe)_2$ on reactive oxygen species production on isolated mice brain mitochondria. Data are expressed as means ± SEM, (n = 5). Results are expressed as percentage of control.* Denotes $p < 0.05$ as compared with the control group. # Denotes $p < 0.05$ as compared with the APAP group

Discussion

Acute exposure to APAP caused marked neurotoxicity in mice and this was associated with changes in mitochondrial functioning and with general oxidative stress. Based on the present results, we suppose that the primary effect of APAP was elevated AST and ALT activities, which is in accordance with a previous report by Fakurazi et al. (2008). The APAP-induced liver damage produces oxidative stress, which affects oxidative balance in the whole organism, including brain tissue, making it more susceptible to oxidative damage. Studies show that APAP can cross the blood–brain barrier (Courad et al. 2001), and we believe that it can be metabolized directly in brain tissue. This hypothesis is supported by several studies (Hansson et al. 1990; Howard et al. 2003) that indicate that rats have an isoform for cytochrome P450 (CYP2E1) responsible for metabolizing APAP into NAPQI. Thus, we believe that after an overdose of APAP, it is distributed to the brain and metabolized directly in the brain, increasing local concentrations of NAPQI.

The increase in lipid peroxidation (Fig. 2) in the brains of the APAP group is in agreement with the findings of other studies (Nencini et al. 2007). The results presented in this article are in agreement with the in vitro antioxidant properties of $(PhSe)_2$ against lipid peroxidation in brain tissue (Rossato et al. 2002; Ghisleni et al. 2003). Accordingly, our data demonstrated a significant in vivo effect of $(PhSe)_2$ in reversing cerebral oxidative stress and significantly decreasing the lipid peroxidation and the oxidation of DCFH (Fig. 3) caused by APAP administration. The treatment with $(PhSe)_2$ 1 h after APAP administration caused a reduction in free radical levels, but this reduction did not show a significant difference from the APAP group.

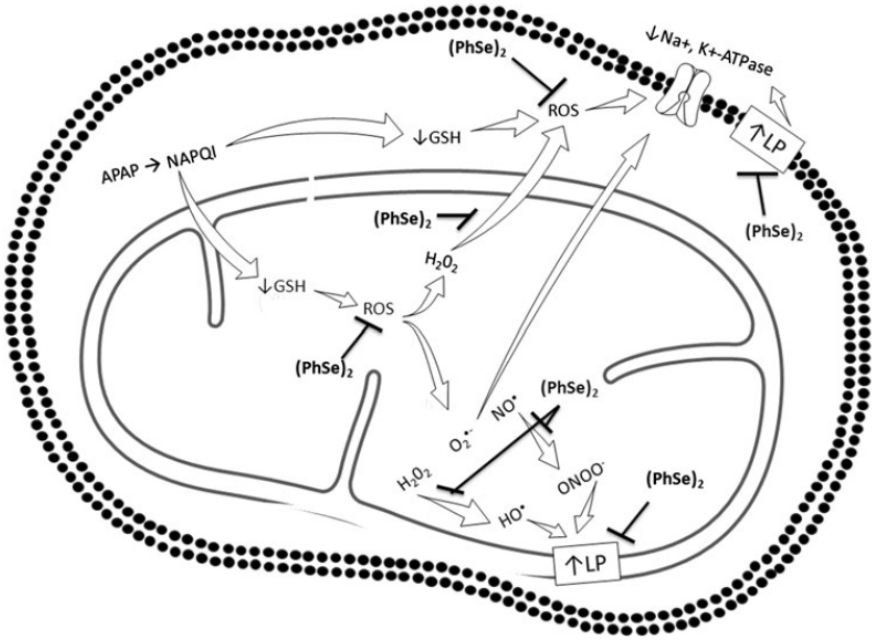
APAP administration caused a significant increase in SOD activity (Fig. 4), which could indicate an excessive production of reactive species. This could be the brain's attempt to reduce the ROS formed by the excess APAP. The administration of $(PhSe)_2$ after APAP reduces SOD levels to the levels of the control group, likely by decreasing O2- production. The exact mechanism of increased SOD activity is unknown, but it may be an adaptive response to excessive oxidative stress (de Freitas et al. 2009) caused by APAP. $(PhSe)_2$ could also have caused a decrease in SOD indirectly via its reduction of oxidative stress. In Addition, in view of the fact that the activity of CAT was unchanged by $(PhSe)_2$ treatment (data not shown), the isolated increase in SOD may reflect further enhancement in oxidative stress via increased H_2O_2 formation.

Membrane lipid peroxidation (Mattson 1998) can alter neurotransmitter homeostasis, ion channel activities, and the function of ATPases. Na^+ , K^+ -ATPase is responsible

for the active transport of sodium and potassium ions in the nervous system, which regulates the cellular Na^+ and K^+ concentrations and their respective gradients across the plasma membrane (Doucet 1988). Therefore, a reduction in the activity of this enzyme may affect neural activity. We found a decrease in the activity of Na^+ , K^+ -ATPase in the APAP group. In fact, this enzyme consumes the greatest part (30–60%) of available ATP in the brain (Bertorello 1995), and the production of ATP in the brain mitochondria of APAP-treated mice may be committed to this function. In addition, thiol groups of this enzyme are highly susceptible to oxidative stress (Yufu et al. 1993), and APAP causes oxidative stress in the brain (Nencini et al. 2007), resulting in the loss of Na^+ , K^+ -ATPase catalytic activity. Other studies have demonstrated that lipid peroxidation and free radicals can lead to inhibition of this enzyme activity in rat brain (Viani et al. 1991; Lees 1993). Accordingly, the present data indicate that $(PhSe)_2$ prevented lipid peroxidation associated with an increase in free radicals, thereby maintaining the structural integrity of the cell membrane and, as a result, abolishing the inhibition of Na^+ , K^+ -ATPase caused by APAP.

Excess NAPQI, which cannot be detoxified by conjugation, can bind to cellular proteins, impair mitochondrial respiration (Meyers et al. 1988), open the MTP pore (Masubuchi et al. 2005), and increase oxidative stress (Bajt et al. 2004). GSH levels can be monitored as a non-specific indicator of cellular toxicity, because a decrease in GSH is indicative of an increased potential for cellular injury. In this study, we observed that GSH levels decreased in the brain of APAP-treated mice, but treatment with $(PhSe)_2$ did not reverse the GSH levels to those of the control group, possibly because $(PhSe)_2$ did not prevent NAPQI formation from APAP.

APAP caused mitochondrial dysfunction, demonstrated by a decrease in membrane potential (Fig. 8), an increase in mitochondrial swelling (Fig. 9), and an increase in ROS production (Fig. 10). These changes may be due to the induction of MPT by oxidative stress caused by the toxic metabolite of APAP (Kim et al. 2003). Mitochondrial protein binding may be responsible for the inhibition of mitochondrial respiration after an APAP overdose (Meyers et al. 1988), which can induce mitochondrial dysfunction and cause mitochondrial oxidative stress and ATP depletion (Jaeschke 1990; Tirmenstein and Nelson 1990). ATP depletion may occur in the brain mitochondria of APAP-treated mice. Further, aggravated by the induction of MPT in mitochondria, acute liver failure can cause accumulation of ammonia in the brain as demonstrated in a study of rat brains, (Panatto et al. 2011) which found a decrease in complexes I and IV of respiratory chain. We believe that this effect on the mitochondrial respiratory chain can aggravate oxidative stress caused by APAP. Moreover, ammonia can cause a decrease in membrane potential and a consequent induction of MPT in the brain, as shown in a study that administered ammonia directly to a culture of astrocytes (Alvarez et al. 2011) This could explain the decrease in cell viability in the group treated with APAP, since previous studies (Kim et al. 2003; Kon et al. 2004; Masubuchi et al. 2005) showed that mitochondrial



Scheme 1 Effects of NAPQI and $(PhSe)_2$ in cell and mitochondria

dysfunction, MPT formation, decrease in mitochondrial respiration, and decrease in ATP synthesis cause cellular apoptosis. There are no studies in the literature showing how reverses mitochondrial dysfunction, and only one article (Puntel et al. 2010), until now, discussed $(PhSe)_2$ and mitochondria, but its aim was to evaluate toxicity in mitochondria. We suggest that the mechanism of $(PhSe)_2$ reversal of mitochondrial dysfunctions $(PhSe)_2$ is by its antioxidant properties. As shown earlier, $(PhSe)_2$ reverses lipid peroxidation (Fig. 2) and ROS generation (Fig. 3) and, consequently, oxidative stress. In short, we believe that $(PhSe)_2$ protects the membrane protein of the mitochondrial membrane against attack by ROS, and this reversed every type of mitochondrial dysfunction seen in the APAP group. Other studies should be undertaken to further define the mechanism by which $(PhSe)_2$ reverses mitochondrial damage.

An earlier study conducted in vivo, which used the same dose of APAP as our study showed that NAPQI does not bind to protein in the brain. However, this study was realized only in the cytosolic fraction and not in mitochondria (Bulera et al. 1996) as we studied here. Therefore, we believe that NAPQI may bind to brain mitochondrial protein, similar to what occurs in the liver (Bulera et al. 1996), though more studies are needed to identify the exact mechanism by which APAP causes toxicity in brain mitochondria.

Scheme 1 shows the mechanism of action of APAP and $(PhSe)_2$ in cells. A toxic dose of APAP caused a decrease in GSH levels by NAPQI and an increase in ROS formation. In cytoplasm, ROS inhibits Na^+ , K^+ -ATPase activity and induces lipid peroxidation, which further inhibits Na^+ , K^+ -ATPase. In mitochondria, an increase in O2 occurs, which can cross the mitochondrial membrane through transition pores and inhibit Na^+ , K^+ -ATPase. An increase in H_2O_2 is also seen, which can be formed from a hydroxyl radical via the fenton reaction and a peroxynitrite radical. These two radicals can cause lipid peroxidation in the mitochondrial membrane. The mechanism of action of $(PhSe)_2$ is to inhibit ROS formation and lipid peroxidation in both the mitochondria and cytoplasm.

As expected, an intraperitoneal dose of APAP caused significant hepatotoxicity and oxidative damage in the brain. Previous studies demonstrated that the oxidative stress eventually affects mitochondrial function, decreases ATP production, and alters mitochondrial permeability. Treatment with $(PhSe)_2$ reversed the oxidative damage and mitochondrial dysfunction induced by APAP in the brains of mice. In conclusion, treatment with $(PhSe)_2$ 1 h after an overdose of APAP caused a reversal of levels of free radicals and normalized antioxidant and mitochondrial functions in the brain. Therefore, $(PhSe)_2$ is an important agent for future studies in the treatment of poisoning with APAP.

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4 CONCLUSÃO

A administração de uma dose tóxica de APAP causou hepatotoxicidade, como relatam outros estudos, desenvolvendo estresse oxidativo e afetando o tecido cerebral. O aumento da atividade das enzimas ALT e AST confirmou que a dose usada de APAP causou dano hepático aos animais. O APAP causou aumento nos marcadores de dano oxidativo no cérebro, aumento da atividade da enzima SOD e inibição na atividade da enzima Na^+-K^+-ATPase. O tratamento com $(PhSe)_2$ foi capaz de reverter as alterações causadas pelo APAP no tecido cerebral. A formação do NAPQI após a dose de APAP foi vista através da diminuição da GSH, o $(PhSe)_2$ não foi capaz de reverter esse parâmetro pois esse composto não interfere na formação do metabólito do APAP. O APAP causou disfunção mitocondrial no cérebro dos animais, sugerindo que possa ter ocorrido a abertura do MPT e o $(PhSe)_2$, devido à sua atividade antioxidante, foi capaz de reverter esses parâmentros. Portanto, o $(PhSe)_2$ mostrou-se capaz de reverter o estresse oxidativo e a disfunção mitocondrial no tecido cerebral devido, principalmente, a sua capacidade antioxidante.

5 PERSPECTIVAS

Tendo em vista os resultados obtidos neste trabalho, as perspectivas para trabalhos posteriores são:

- Determinar o mecanismo pelo qual o $(PhSe)_2$ foi capaz de reverter a disfunção mitocondrial causada pelo APAP.

- Verificar a ligação do NAPQI com proteínas da fração mitocondrial no tecido cerebral.

- Realizar um estudo mostrando a distribuição no $(PhSe)_2$ nas diferentes áreas do cérebro

- Adicionar NAC ao tratamento com o $(PhSe)_2$ e realizar um estudo comparativo entre os trabalhos

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