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EFETIVIDADE DO $(PhSe)_2$ EM PROTEGER CONTRA A TOXICIDADE DO $HgCl_2$

DISSERTAÇÃO DE MESTRADO

Tiago da Luz Fiuza

Santa Maria, RS, Brasil
2014

EFETIVIDADE DO $(PhSe)_2$ EM PROTEGER CONTRA A TOXICIDADE DO $HgCl_2$

Tiago da Luz Fiuza

Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de **Mestre em Bioquímica Toxicológica**.

Orientadora: Profª. Drª. Maria Ester Pereira

Santa Maria, RS, Brasil
2014

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A Comissão Examinadora, abaixo assinada,
aprova a Dissertação de Mestrado

EFETIVIDADE DO $(PhSe)_2$ EM PROTEGER CONTRA A TOXICIDADE DO $HgCl_2$

elaborada por
Tiago da Luz Fiuza

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

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Santa Maria, 13 de junho de 2014

DEDICATÓRIA

Dedico esta dissertação:

Aos meus pais Antonio e Roceli, aos meus irmãos Tony e Tamara e a minha namorada Veronica, pessoas que para mim sempre foram fonte de motivação e inspiração.

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RESUMO

Dissertação de Mestrado

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica

Universidade Federal de Santa Maria, RS, Brasil

Efetividade do $(PhSe)_2$ em proteger contra a toxicidade do $HgCl_2$

AUTOR: Tiago da Luz Fiuza

ORIENTADOR: Maria Ester Pereira

LOCAL E DATA DA DEFESA: Santa Maria, 13 de junho de 2014

O mercúrio (Hg) é um metal não essencial, ou seja, sem função biológica e também um poluente ambiental originário de diversas fontes, principalmente de áreas de exploração de minérios e atividade industrial intensa. É bem conhecido que o Hg possui uma alta afinidade por grupamentos tiois (SH) ocasionando diversas alterações bioquímicas que podem induzir ao estresse oxidativo. O selênio (Se) por sua vez, é um elemento traço essencial, pois está envolvido em diversos processos metabolicos, principalmente naqueles envolvidos no sistema de defesa antioxidante. O disseleneto de difenila $(PhSe)_2$ é um composto orgânico de selênio com propriedades farmacológicas, das quais destacam-se suas propriedades antioxidantes e antiinflamatórias. O objetivo deste trabalho foi avaliar o efeito protetor do $(PhSe)_2$ contra a intoxicação induzida por cloreto de mercúrio ($HgCl_2$). Para isso foram utilizados camundongos *Swiss albinos* (17-25 g) os quais foram tratados por cinco dias consecutivos com $(PhSe)_2$ 5,0 mg/kg ou óleo de canola por via oral, e nos cinco dias seguintes com $HgCl_2$ 5,0 mg/kg ou salina, por injeção subcutânea. Os animais foram sacrificados 24h após a última administração de $HgCl_2$ e as amostras de tecido sanguíneo, renal e hepático foram coletadas para a analise da atividade da enzima δ-aminolevulinato desidratase (δ-ALA-D). A atividade da ALT e AST séricas e os níveis séricos de uréia e creatinina foram avaliados como parâmetros de toxicidade hepática e renal, respectivamente. Também foram avaliados os parâmetros oxidativos, tiois totais, tiois não protéicos e os níveis de espécies reativas ao ácido tiobarbitúrico. Foram determinados também os níveis de Hg e Se nos tecidos. Os animais expostos ao mercúrio apresentaram uma inibição da atividade da enzima δ-ALA-D sanguínea, a qual foi parcialmente prevenida pelo $(PhSe)_2$. Quanto aos parâmetros de toxicidade hepática e renal, os animais expostos ao Hg apresentaram uma inibição na atividade da enzima ALT e um aumento nos níveis de creatinina, o qual foi prevenido totalmente pelo $(PhSe)_2$. Em relação aos parâmetros oxidativos, foi observado uma diminuição nos níveis de espécies reativas ao acido tiobarbiturico (TBARS) e um aumento nos níveis de tiois não protéicos no tecido renal. Este aumento foi parcialmente prevenido pelo $(PhSe)_2$. Os animais expostos ao Hg apresentaram níveis elevados deste metal no rim, fígado e sangue. Os animais expostos ao Hg pré-tratados com $(PhSe)_2$, apresentaram níveis diminuídos de Hg no sangue e aumentados no fígado em relação aos animais tratados somente com Hg. Considerando os resultados apresentados e tendo em vista as propriedades antioxidantes do $(PhSe)_2$ e os mecanismos envolvidos na toxicidade do mercúrio, acredita-se que o (PhSe)2 pode vir a ser um agente em potencial contra a intoxicação por mercúrio.

ABSTRACT

Dissertation of Master's Degree

Post-Graduate Course in Biological Science - Toxicological Biochemistry

Federal University of Santa Maria, RS, Brazil

Effectiveness of $(PhSe)_2$ in protect against the $HgCl_2$ toxicity

AUTHOR: Tiago da Luz Fiuza

ADVISOR: Maria Ester Pereira

DATE AND PLACE OF THE DEFENSE: Santa Maria, June, 13, 2014

Mercury (Hg) is a non-essential metal without biological function and also an environmental pollutant originating from various sources, especially from areas of mineral exploration and intense industrial activity. It is well known that Hg has a high affinity for thiol groups (SH) causing several biochemical changes that induce oxidative stress. On the other hand, selenium (Se) is an essential trace element involved in many processes in metabolism, especially which are related to antioxidant defense system. The diphenyl diselenide $(PhSe)_2$ is an organic selenium compound with pharmacological properties, such as antioxidant and anti-inflammatory. The objective of this study was to evaluate the protective effect of $(PhSe)_2$ against intoxication induced by mercuric chloride ($HgCl_2$) poisoning. For this Swiss albino mice (17-25g) were treated for five consecutive days with $(PhSe)_2$ 5.0 mg/kg or canola oil by oral gavage and with $HgCl_2$ 5.0 mg/kg or saline by subcutaneous injection for more five days. The animals were sacrificed 24 hours after the last administration of $HgCl_2$ and samples of blood, kidney and liver tissue were collected for δ-aminolevulinate dehydratase (δ-ALA-D) activity assays. The activity of serum AST and ALT and serum urea and creatinine levels were assessed as parameters of hepatic and renal toxicity, respectively. Oxidative parameters, total thiols, non-protein thiols and the levels of thiobarbituric acid species were also evaluated. Levels of Hg and Se were determined in tissues. The animals exposed to mercury showed an inhibition of blood δ-ALA–D activity, which was partially prevented by $(PhSe)_2$. Regarding the parameters of liver and kidney toxicity, animals exposed to Hg showed an inhibition of ALT activity and an increase in creatinine levels, been this last completely prevented by $(PhSe)_2$. On oxidative parameters evaluated, a decrease in thiobarbituric acid reactive specimens (TBARS) levels and an increase in non-protein thiols in the renal tissue were observed. This increase was partially prevented by $(PhSe)_2$. Animals exposed to Hg showed high levels of this metal in the kidney, liver and blood. Animals exposed to Hg and pre-treated with $(PhSe)_2$ showed a decrease of Hg levels in the blood and an increased in the liver when compared to those only treated with Hg. Therefore, considering the results presented and the antioxidant properties of $(PhSe)_2$ and the mechanisms underlying the toxicity of mercury, it is believed that $(PhSe)_2$ may be a potential agent against mercury poisoning.

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RESULTADOS

LISTA DE ABREVIAÇÕES

ALT: alanina aminotransferase

AST: aspartato aminotransferase

ANOVA: análise de variância (analysis of variance)

°C: grau Celsius

DNA: ácido desoxirribonucléico

δ-ALA-D: delta-aminolevulinato desidratase

EO: estresse oxidativo

ERO: espécies reativas de oxigênio

GPx: glutationa peroxidase

Gr: glutationa redutase

GSH: glutationa reduzida

h: hora

Hg: mercúrio

Hg°: mercúrio metálico; mercúrio elementar

m: mili

M: molar

µ: micro

min: minuto

n: número de repetições

NPSH: tióis não protéicos

p: nível de significância

PBG: porfobilinogênio

pH: potencial hidrogeniônico

rpm: rotações por minuto

s.c.: subcutânea; subcutaneamente

S.E.M.: standard error of mean (erro padrão da média)

SH: grupos sulfidrílicos

SOD: superóxido dismutase

TCA: ácido tricloroacético

TSH: tióis totais

U: unidades

v/v: volume/volume

Zn: zinco

Sumário

APRESENTAÇÃO

Esta dissertação está descrita na seguinte forma: primeiramente são apresentados a introdução e os objetivos. A seguir, os resultados e a discussão são apresentados no item manuscrito. O item conclusão encontrado no final desta dissertação, apresenta interpretações gerais sobre o manuscrito contido neste trabalho. As referências bibliográficas apresentadas no final da dissertação referem-se somente as citações que aparecem no item introdução.

1. INTRODUÇÃO

1.1. Mercúrio

O mercúrio é um metal divalente pertencente à família 2B, ou família do Zinco (Zn), da tabela periódica. Este metal não essencial, ou seja, sem função biológica, encontra-se no estado líquido mesmo em temperatura ambiente. Devido ao aumento das atividades industriais e de mineração este metal acaba sendo liberado no meio ambiente. Intensas atividades vulcânicas e geotermais também contribuem para a liberação de mercúrio para o ambiente, sendo estas, as formas naturais de liberação do mercúrio para a biosfera (Li et al. 2009; WHO et al. 1991). O mercúrio pode ser encontrado nas formas elementar (Hg^0), orgânico e inorgânico (Hg^{2+}), sendo que destes últimos destacam-se o metilmercúrio (MeHg) e o cloreto de mercúrio ($HgCl_2$) como seus principais exemplos, respectivamente (UNEP, 2002; KLAASSEN, 1996). Todas as formas de mercúrio causam efeitos tóxicos em diversos tecidos e órgãos. Contudo, seus efeitos tóxicos, distribuição e toxicocinética vão depender da forma química, nível e tempo em que o organismo foi exposto (CLARKSON, 2002; FARINA et al. 2011). O mercúrio elementar e o mercúrio orgânico atingem principalmente o sistema nervoso e o pulmonar, enquanto a forma inorgânica atinge principalmente o sistema renal e hepático (BERLIN et al. 2007; CECCATELLI et al. 2010; FARINA et al. 2011).

1.2. Toxicidade do mercúrio inorgânico

Dentre os diversos danos que esta forma de mercúrio pode causar ao organismo, destacam-se os danos ao sistema renal, uma vez que o rim é um órgão altamente perfundido e, por tanto, constantemente exposto a agentes tóxicos. Além do mais, a eliminação urinaria é a principal rota de eliminação de metais tóxicos (FOWLER, 1992; AU, 2004). A ligação deste metal com grupamentos SH de proteínas, peptídeos e aminoácidos leva a necrose do epitélio renal, acompanhado de proteinúria e glomerulonefrite (GOYER, 1996). Diversos estudos sugerem que um importante mecanismo envolvido no dano renal induzido pela forma

inorgânica do mercúrio envolve a indução do estresse oxidativo. A alta afinidade do mercúrio com grupamentos SH nos componentes celulares induz a diminuição de outros grupamentos SH (especialmente GSH) do interior da célula, com isso aumenta a predisposição das células tubulares proximais ao estresse oxidativo (FUKINO et al. 1984). Essa forma de mercúrio também está relacionada a danos do sistema hepático, podendo causar alterações histopatológicas, vacuolização citoplasmática, cariorrexia, cariólise, picnose e necrose centro tubular (KUMAR et al. 2005).

Apesar do cloreto de mercúrio não atravessar facilmente a barreira hemato-encefálica como o mercúrio orgânico, o sistema nervoso também pode ser afetado pelo cloreto de mercúrio uma vez que essa forma de mercúrio pode ser formada nesse órgão como um metabólito de outras formas de mercúrio, onde pode permanecer durante anos (VAHTER et al. 1994).

Os efeitos tóxicos causados pela exposição ao Hg estão relacionados ao fato deste metal interferir em diferentes processos bioquímicos do organismo, devido a sua grande afinidade por grupamentos sulfidrílicos (SH) (ROONEY, 2007). Também, Agarwal e colaboradores (2010) demonstraram que ratos expostos ao Hg apresentaram uma diminuição na atividade das enzimas de defesa antioxidante: superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx) e glutationa redutase (GR), em tecido hepático, renal e cerebral.

1.3. Estresse Oxidativo

Tanto em condições fisiológicas como patológicas, o metabolismo celular produz continuamente espécies reativas de oxigênio (ERO), através da respiração e outras atividades metabólicas (HALLIWELL, 1994; AZBILL et al. 1997; MORGAN et al. 2007). Essas ERO são neutralizadas pelo sistema de defesa antioxidante, que pode ser enzimático ou não enzimático. Porém, quando ocorre um aumento na produção de espécies reativas ocorre também um desequilíbrio no sistema de defesa antioxidante, evento este denominado estresse oxidativo (RODRIGUEZ-MARTINEZ et al. 2000; SANTAMARÍA et al. 2003; DAWSON & DAWSON, 1996; HALLIWEL & GUTTERIDGE, 2007). O sistema antioxidante enzimático atua de uma forma interligada, onde o produto de uma enzima serve de substrato para outra até que o radical livre seja neutralizado (Figura 1) (YAO & KESHAVAN, 2011).

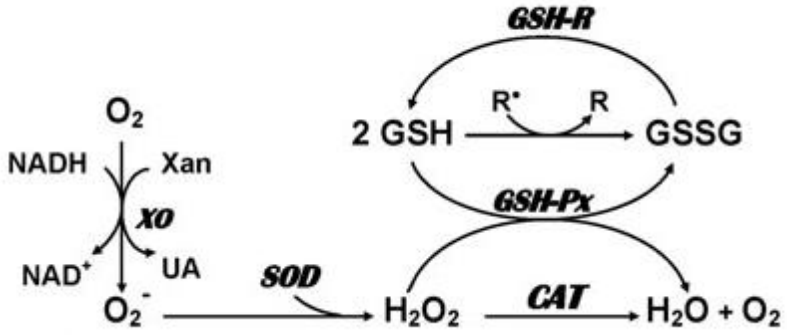

Figura 1. Mecanismo envolvendo a neutralização de radicais livres através de antioxidantes enzimáticos em células de mamíferos (adaptado de YAO & KESHAVAN, 2011).

Além do sistema antioxidante enzimático citado anteriormente, diversas substâncias, tais como as vitaminas C e E, flavonóides e o tripeptídeo glutationa (GSH), representam o grupo não enzimático de substâncias com atividade antioxidante, que diferentemente do sistema de defesa enzimático, atua neutralizando as espécies reativas através de uma interação direta (REISCHL et al., 2007).

A diminuição das defesas antioxidantes, tanto enzimáticas como não-enzimáticas juntamente com o aumento das espécies reativas, leva ao aumento da peroxidação lipídica e danos no DNA, gerando alterações e danos celulares (FARINA et al. 2011). A interação entre o Hg e grupamentos SH diminui a capacidade de agentes antioxidantes em neutralizar espécies reativas, levando assim a um quadro de estresse oxidativo (ROONEY, 2007). Outros metais tóxicos como o cádmio e o chumbo também podem causar essas alterações (LIU et al. 2010; LUCHESE et al. 2007).

Diversas pesquisas são desenvolvidas com o objetivo de testar diferentes compostos que consigam combater ou pelo menos diminuir o estresse oxidativo gerado por substâncias tóxicas. Alguns desses estudos mostram que a suplementação com compostos como o selênio (JIHEN et al., 2009), flavonóides (PROCHÁZKOVÁ et al. 2011), vitamina E (AGARWAL et al. 2010), vitamina C (AKSOY et al. 2005), cisteína (JOSHI et al. 2011) e zinco (JEMAI et al. 2007) podem ter efeitos relevantes.

1.4. Selênio

O selênio (Se) é um elemento traço pertencente ao grupo 16 da tabela periódica, conhecido como o grupo dos calcogênios. Pode ser encontrado em diferentes estados de oxidação, tais como selênio elementar (Se^0), selenito (Se^{+4}), selenato (Se^{+6}) e seleneto (Se^{-2}), e pode ser encontrado na natureza tanto na forma orgânica (selenocisteína, selenocistina e selenometionina), como na forma inorgânica (selenito e selenato) (BARCELOUX, 1999).

Por ser considerado um elemento traço essencial para a saúde (PAPP et al. 2007), são requeridas quantidades adequadas deste elemento para a manutenção da saúde, visto que quantidades insuficientes ou excessivas podem ocasionar deficiência ou toxicidade, respectivamente (RAYMAN, 2000). O selênio está amplamente distribuído em alimentos como, alho, cebola, castanha-do-pará, brócolis, cogumelos, cereais, pescado, ovos e carnes (DUMONT et al. 2006).

O Se desempenha importante papel em diversas vias metabólicas, que incluem o crescimento celular, funções imunológicas, metabolismo dos hormônios tireoidianos, mas principalmente nas vias que envolvem o sistema de defesa antioxidante. No sistema antioxidante, este elemento esta presente em enzimas como a glutationa peroxidase (GPx) e a tioredoxina redutase (TRx), na forma de selenocisteína no sítio ativo (STAZI & TRINTI, 2008; FLOHÉ et al. 1973; HOLMGREN, 1985).

Sabe-se que o Se possui efeito na distribuição do Hg no organismo, e esse efeito pode reduzir a toxicidade causada por este metal (GOYER et al., 1995). Uma vez que o Se desempenha um importante papel na defesa antioxidante do organismo, existe uma crescente busca pelo entendimento do seu papel biológico, em particular o seu uso como agente terapêutico (PAPP et al. 2007). O mecanismo exato da interação entre o Se e o Hg, e como o Se interfere na toxicidade do Hg, ainda não é completamente compreendido. Porém, existem alguns mecanismos propostos que sugerem o efeito protetor do Se em intoxicação por Hg: (1) na presença de Se o mercúrio é redistribuido de órgãos mais sensíveis como os rins para órgãos menos sensíveis como os músculos, (2) competição entre sítios de ligação entre o Se e o Hg, pois sabe-se que tanto o selênio quanto o mercúrio possuem alta afinidade por grupamentos SH de aminoácidos de proteínas carreadoras e também outros sítios de ligação (3) formação de um complexo Se-Hg, pois a administração simultânea de $HgCl_2$ e selênio na forma selenito altera a ligação do Hg às seleno-proteinas, (4) conversão da forma tóxica do Hg em outras formas menos tóxicas e (5) prevenção do dano oxidativo pois o Se é um

componente intrínseco da enzima antioxidante glutationa peroxidase (CUVIN-ARALAR et al. 1991).

Compostos orgânicos de Se tem sido alvo de diversos estudos, em virtude de suas propriedades farmacológicas e também por apresentarem uma melhor biodisponibilidade e menor toxicidade em comparação com as formas inorgânicas desse elemento (PARNHAM & GRAF, 1991; NOGUEIRA et al. 2004; NARRAJJI et al. 2007).

O disseleneto de difenila $(PhSe)_2$ (Figura 2) é um composto orgânico de Se com diversas propriedades farmacológicas, entre elas, antidepressiva (SAVEGNAGO et al. 2008) e antiinflamatória (NOGUEIRA et al. 2003). Esse composto orgânico de Se também apresenta pronunciada ação como antioxidante na redução da peroxidação lipídica causada por diferentes agentes e em diferentes tecidos (MEOTI et al. 2004; ROSSATO et al. 2002; NOGUEIRA et al. 2004; SANTOS et al. 2004; BORGES et al. 2006; POSSER et al. 2009).

O $(PhSe)_2$ reverteu os danos oxidativos induzidos pelo cádmio em fígado, rim, sangue e cérebro de camundongos (SANTOS et al. 2004). Em estudos realizados por Brandão e colaboradores (2008), observou-se que o pré-tratamento com $(PhSe)_2$ foi efetivo em proteger contra as alterações hematológicas causadas pelo $HgCl_2$.

Contudo, compostos orgânicos de selênio possuem "dupla face", pois em baixas doses esses compostos podem apresentar efeitos benéficos e em doses elevadas apresentam efeitos tóxicos. Porem a dose limite para esses efeitos opostos ainda não é estabelecida (NOGUEIRA & ROCHA, 2010).

De Freitas et al. (2009) demonstraram que o $(PhSe)_2$, em doses de 0,4 e 1,0 mg/Kg protegeu contra danos oxidativos em tecido renal, hepático e cerebral em camundongos expostos ao metil mercúrio. Entretanto Brandão e colaboradores (2010) demonstraram que o (PhSe)2 administrado concomitantemente ao $HgCl_2$ potencializou os efeitos nefrotóxicos induzidos pelo Hg. Convém mencionar que estes dois estudos diferem não somente na dose de $(PhSe)_2$ utilizada mas também na forma de mercúrio (Orgânica X Inorgânica) utilizada.

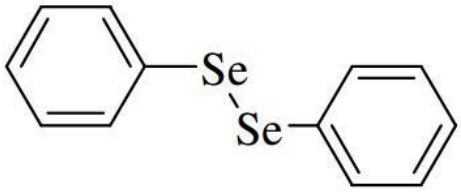

Figura 2. Disseleneto de difenila

1.5. Marcadores de efeito tóxico

A Ureia e a creatinina são os principais indicadores de dano renal agudo sendo muito utilizados no diagnóstico clínico (EDELSTEIN, 2008). A ureia é o principal produto do catabolismo das proteínas, e serve de mecanismo para excreção da amônia. A creatinina por sua vez é formada pelo metabolismo normal da musculatura devido a degradação da fosfocreatinina. Ambos os metabólitos são excretados pelos rins, por tanto, o aumento de seus níveis no sangue indica falha na função renal (RAVEL, 1997). Os níveis plasmáticos de creatinina são um indicador mais sensível que os níveis de ureia durante a fase inicial de danos renais, uma vez que os níveis de ureia aumentam somente depois de estabelecido danos no parênquima do tecido renal (GILBERT et al. 1989). Alguns estudos tem relacionado a toxicidade renal causada pelo mercúrio com o aumento sanguíneo de ureia e creatinina em ratos em diferentes períodos de desenvolvimento (PEIXOTO & PEREIRA, 2007; FRANSCISCATO et al. 2011; AGARWAL et al. 2010).

Estudos tem mostrado que o $HgCl_2$ também causa alterações na atividade da alanina aminotransferase (ALT), possibilitando a utilização dessa enzima como marcador de hepatotoxicidade (VEDAVATHI et al. 2004). Recentemente, verificou-se uma inibição da ALT sérica de ratos jovens expostos ao $HgCl_2$, onde foi sugerido uma provável interação do metal com grupamentos sulfidrílicos presentes na estrutura da enzima (PEIXOTO et al. 2007; FRANCISCATO et al. 2011; MORAES-SILVA et al. 2012).

Outro exemplo de enzima que possui grupamentos -SH em seu sítio ativo é a δ-aminolevulinato desidratase (δ-ALA-D). Esta enzima, que está presente na maioria dos tecidos, tem como substrato o ácido delta aminolevulínico e catalisa a condensação assimétrica de duas moléculas deste ácido para formar o composto monopirrólico porfobilinogênio (Figura 3), participando assim da rota da síntese do heme (GIBSON et al. 1955; SASSA et al. 1989). A inibição dessa enzima pode levar ao acúmulo do ácido δ-aminolevulínico, que possui efeito pró-oxidante, levando a geração de radicais livres e, consequentemente, aumenta o dano oxidativo nos componentes celulares (PEREIRA et al. 1992; BECHARA, 1996).

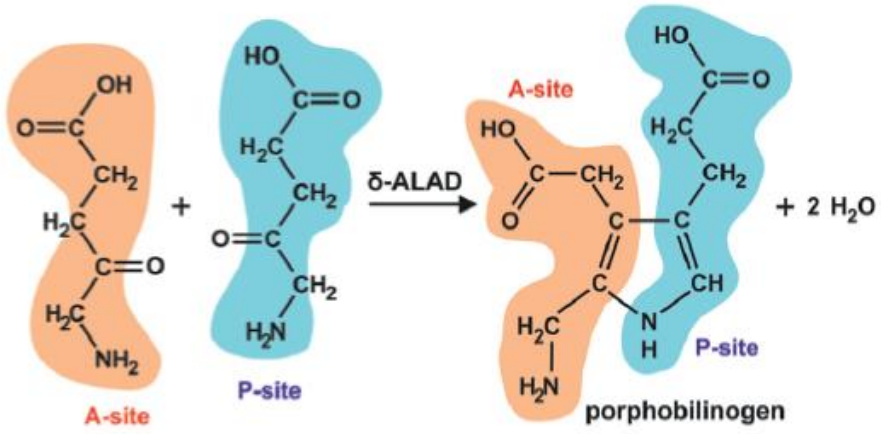

Figura 3. Síntese de porfobilinogênio (PBG) a partir de duas moléculas do ácido 5-aminolevulínico (δ-ALA) (adaptado de ROCHA et al. 2012)

Em mamíferos, esta enzima requer o zinco (Zn) como co-fator no seu sítio ativo, o qual possui um único sítio de ligação do zinco com três resíduos de cisteína (JAFFE, 1995; GODWIN, 2001). Estes grupamentos sulfidrílicos estão envolvidos na coordenação essencial do íon Zn, e a proximidade entre eles torna a enzima particularmente sensível a oxidação. O íon Zn esta envolvido também na estabilização destes grupos sulfidrílicos e a sua remoção pode acelerar o processo de auto-oxidação da enzima perdendo assim sua capacidade catalítica. Devido a proximidade entre os grupamentos SH presentes no sítio ativo, a δ-ALA-D torna-se bastante sensível a oxidação por metais divalentes como o Hg ou o chumbo (Pb), por exemplo, pois esses metais possuem a capacidade de remover o Zn presente no sítio ativo e ligarem-se aos grupamentos sulfidrílicos, fazendo com que esta perca sua atividade catalítica (MARKHAM et al. 1993; SARAIVA et al. 2012). Diversos estudos tem mostrado que tanto animais jovens (PEIXOTO et al. 2007) como adultos (OLIVEIRA et al. 2014), quando expostos ao mercúrio apresentam uma inibição da atividade da enzima δ-ALA-D, que por sua vez, pode ser usada como marcador de exposição ao Hg. Além disso, é bem conhecido seu papel como marcador da exposição ao chumbo em humanos. Devido às características apresentadas, esta enzima torna-se um importante marcador de efeitos tóxicos do Hg (ROCHA et al. 2012).

Tendo em vista as propriedades antioxidantes do $(PhSe)_2$ e os possíveis mecanismos envolvidos na toxicidade do Hg, cria-se a perspectiva de um tratamento preventivo que possa impedir ou ao menos amenizar os efeitos tóxicos induzidos pela exposição a esse metal, com isso propor a possível utilização do $(PhSe)_2$ como uma nova proposta terapêutica para a intoxicação por Hg.

2. OBJETIVOS

2.1. Objetivo geral

Avaliar a possível prevenção exercida pelo $(PhSe)_2$ contra a toxicidade induzida pelo Hg em camundongos.

2.2. Objetivos específicos

Avaliar:

- ✓ a atividade da δ-aminolevulinato desidratase (δ-ALA-D) em rins, fígado e sangue de camundongos como marcador de efeito tóxico;
- ✓ a função renal através da análise dos níveis séricos de ureia e creatinina;
- ✓ a função hepática por meio da atividade da alanina aminotranferase (ALT) e aspartato aminotransferase (AST) séricas;
- ✓ os parâmetros oxidativos não enzimáticos como tióis totais e não proteicos e TBARS de rins e fígado;
- ✓ os níveis de Se e Hg em rins, fígado e sangue.

3. DESENVOLVIMENTO

Os resultados que fazem parte desta dissertação estão apresentados sob a forma do manuscrito intitulado *"**Effetiveness of ($PhSe_2$ in protect against $HgCl_2$ toxicity**"*. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências (do manuscrito), encontram-se no próprio manuscrito, o qual foi aceito para publicação na revista *Journal of Trace Elements in Medicine and Biology* (JTEMB-D-14-00100R1). A seguir estão apresentadas as conclusões deste trabalho, as perspectivas que surgiram a partir deste, e as referências bibliográficas correspondentes as citações feitas na introdução da dissertação.

3.1. Manuscrito

Efetividade do $(PhSe)_2$ em proteger contra a toxicidade do $HgCl_2$

Effetiveness of $(PhSe)_2$ in protect against HgCl2 toxicity

Tiago da Luz Fiuza, Cláudia Sirlene Oliveira, Michael da Costa, Vitor Antunes Oliveira, Gilson Zeni, Maria Ester Pereira

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Effectiveness of $(PhSe)_2$ in protect against the $HgCl_2$ toxicity

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Abstract

This work investigated the preventive effect of diphenyl diselenide [$(PhSe)_2$] on renal and hepatic toxicity biomarkers and oxidative parameters in adult mice exposed to mercury chloride ($HgCl_2$). Selenium (Se) and mercury (Hg) determination was also carried out. Mice received a daily oral dose of $(PhSe)_2$ (5.0 mg/kg/day) or canola oil for five consecutive days. During the following five days, the animals were treated with a daily subcutaneous dose of $HgCl_2$ (5.0 mg/kg/day) or saline (0.9%). Twenty four hours after the last $HgCl_2$ administration, the animals were sacrificed and biological material was obtained. Concerning toxicity biomarkers, Hg exposure inhibited blood δ-aminolevulinic acid dehydratase (δ-ALA-D), serum alanine aminotransferase (ALT) activity and also increased serum creatinine levels. $(PhSe)_2$ partially prevented blood δ-ALA-D inhibition and totally prevented the serum creatinine increase. Regarding the oxidative parameters, Hg decreased kidney TBARS levels and increased kidney non-protein thiol levels, while $(PhSe)_2$ pre-treatment partially protected the kidney thiol levels increase. Animals exposed to $HgCl_2$ presented Hg content accumulation in blood, kidney and liver. The $(PhSe)_2$ pre-treatment increased Hg accumulation in kidney and decreased in blood. These results show that $(PhSe)_2$ can be efficient in protecting against these toxic effects presented by this Hg exposure model.

Key words: Selenium; Diphenyl diselenide; Mercury; δ-aminolevulinic acid dehydratase; Se and Hg levels.

1. Introduction

Mercury (Hg) is a nonessential metal which can be found in elemental, organic and inorganic forms[1,2]. Studies have shown that mercury chloride ($HgCl_2$), an inorganic form of this element [3,4], can cause damage to the organs of several known animals, such as mice and rats [5-7]. Its toxicity is mainly expressed in renal tissue, since the kidneys are the primary route to toxic metal elimination [8,9], not to mention Hg has high affinity by sulfhydryl groups binding to biomolecules containing this radical [10]. Likewise, Hg interaction with sulfhydryl groups decreases the ability of antioxidant agents, such as glutathione (GSH), in neutralizing reactive oxygen species (ROS), consequently leading to lipid peroxidation [11].

Several drugs and compounds have been tested with the objective of reducing Hg damage [6,11]. Previous studies performed by our research group have shown the ability of zinc and copper, essential trace elements, in protecting against the Hg toxicity [12-18].

Another important trace element in the metabolism is selenium (Se). Like Hg, Se can be found in elemental, organic and inorganic forms [19]. Se participates in various metabolic routes, especially those that involve the antioxidant system; for example, Se is present in active sites of antioxidant enzyme glutathione peroxidase (GPx) [20,21]. It is also known that Se alters the Hg distribution in the organism, and due to this effect Hg toxicity can be reduced [22]. Moreover, Se can reduce the Hg toxicity through the prevention of oxidative damage [23].

Diphenil disselenide $(PhSe)_2$ is an organoselenium compound with various pharmacologic activities such as antidepressant [24] and anti-inflammatory [25]. This compound has shown antioxidant activity against lipid peroxidation induced by different agents in different tissues [26-31]. Moreover, $(PhSe)_2$ was shown to be efficient in protecting hematological alterations induced by Hg[32].

Thus, considering that Se is an essential element with beneficial antioxidant property, the present study evaluated the protective effect of $(PhSe)_2$ against renal and hepatic damage induced by $HgCl_2$ in mice, as well as on oxidative parameters. Still, Hg and Se levels in kidney, liver and blood were determined to evaluate the influence of the element redistribution.

2. Materials and methods

2.1 Chemicals

Reagents used in this study were obtained from Sigma Chemical Co. (St. Louis, MO) and standard commercial suppliers. Commercial kits were used for the biochemical dosages, those of which were obtained from Kovalent do BrasilLtda (Rio de Janeiro - Brazil) and LabtestDiagnóstica S.A. (Minas Gerais - Brazil). $(PhSe)_2$ was prepared according to the method described by Paulmier [33].

2.2 *Animals*

Adult male Swiss mice were obtained from the animal house of the Federal University of Santa Maria and transferred to our breeding colony. They were kept on a 12 h light/dark cycle, a controlled temperature of 22 ± 2˚ C and with free access to food (Guabi, RS, Brazil) and water. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, the Federal University of Santa Maria, Brazil (Process number 039/2014). All efforts were made to minimize the number of animals used and their suffering.

2.3 *Experimental protocol*

The animals were weighed and treated for five consecutive days with $(PhSe)_2$ (5.0mg/kg) or canola oil (oil, vehicle) by oral gavage (v.o.). During the following five days, the animals were weighed and treated with $HgCl_2$ (5.0mg/kg) or saline (sal, vehicle) by subcutaneous injection (s.c.). Mice were distributed in four experimental groups: oil-sal (control group), Se-sal (Se group), oil-Hg (Hg group) and Se-Hg (Se-Hg group). Twenty four hours after the last $HgCl_2$ administration, mice were weighed and killed by cardiac puncture. Blood, renal and hepatic tissues samples were collected and processed according to the technique to be performed.

2.4 *Tissue preparation*

To obtain the serum for urea and creatinine levels, aspartate (AST) and alanine aminotransferase (ALT) activities determination, blood samples were collected in tubes without anticoagulant and centrifuged at 2000 x *g* for 10 min at room temperature. For the δ-ALA-D activity assay, blood samples were collected in tubes with heparin and then transferred to a recipient with distilled water, in 1:4 (v/v) proportions, under constant agitation on ice bath for 10 min to full hemolysis. Kidneys and liver were quickly removed and placed on ice and homogenized in 5 and 7 volumes, respectively, of Tris-HCl buffer (10 mM, pH: 7,4) to obtain the homogenate. The homogenates were centrifuged at 8000 x *g* for 30 min at 4˚C and the low-speed supernatants (S1) were separated and used for δ-ALA-D activity and TBARS level determination. Furthermore, a portion of blood, kidney and liver was removed and frozen at -20^{o}C until Hg and Se level determination.

2.5 Biochemical Determinations

All biochemical determinations were carried out by spectrophotometry using a Biospectro – SP-22 spectophotometer.

2.5.1δ-ALA-D activity

This technique was performed according to Sassa [34] by measuring the rate of product formation (porphobilinogen, PBG), as previously described by Peixoto et al. [14]. The incubation system was previously pipetted and after addition of 100 µL of hepatic S1 or 200 µL of blood hemolyzed or renal S1, the incubation was initiated and carried out for 30, 60 and 90 min for liver, blood and kidney, respectively, at 39 ˚C. The reaction was then stopped by the addition of trichloroacetic acid (TCA) 10% containing $HgCl_2$ 0.05 M and the PBG formation was measured with Ehrlich`s reagent, using the molar absorption coefficient of 6.1 X 10^4 for Ehrlich-PBG salt. The absorbance was determined spectrophotometrically at 555 nm, and the specific enzymatic activity was expressed as nmol PBG/h/mg protein.

2.5.2 ALT and AST activity

ALT activity was determined in a medium containing buffer 55.8 mM, α-ketoglutaric acid 1.67 mM, L-alanine 83.3 mM, sodium azide 12.8 mM and 25 mL of serum incubated at 37 °C for 30 min. The reaction was stopped by adding HCl 0.45 mM. The color reactive (2.4-dinitrophenylhydrazine 0.45 mM) was then added and the medium was incubated for 20 min at room temperature. The color was intensified by NaOH 0.33 mM and the absorbance was determined spectrophotometrically at 505 nm. The activity (U/L) was calculated by comparing with a calibration curve utilizing sodium pyruvate as standard. The AST activity was determined similarly to ALT enzyme, except that the L-aspartic acid 83.3 mM was used as a substrate and that the medium was incubated at 37 °C for 60 min.

2.5.3 Urea levels

Urea levels were determined using a Labtest commercial kit. 10 µL of serum sample was added to a medium containing phosphate buffer (19.34 mM pH 6.9), sodium salicylate (58.84 mM), sodium nitroprusside (3.17 mM), and urease (≥12.63 UK/L) for 5 min at 39 °C. The reaction was then stopped by adding oxidant solution (final concentrations: NaOH 0.07 M and sodium hypochlorite 3.01 mM) and incubated for another 5 min for color development. The absorbance was determined at 600 nm.

2.5.4 Creatinine Levels

Creatinine levels were determined using a Labtest commercial kit. The estimation of creatinine serum levels was carried by measuring the quantity of the product formed (creatinine picrate), and using creatinine as standard. 50µL of serum sample was added to a medium containing picric acid (20.2 mM) and NaOH (145.4 mM) and incubated at 37ºC and the absorbance was measured spectrophotometrically at 510 nm.

2.5.5 TBARS assays

The lipid peroxidation was determined according to the method described by Ohkawa et al. [35] through the measurement of the thiobarbituric acid-reactive species (TBARS). 200µL of renal or hepatic S1 was incubated with 300 µL of thiobarbituric acid (TBA) (0.8%), 200 µL of SDS (8.1%) and 500 µL of acetic acid buffer (2.5M, pH 3.4) for 2 h at 95 °C. For Blood TBARS determination, an aliquot of heparinized blood was homogenized in trichloroacetic acid (TCA) 40%, in 2:1 (v/v) proportions, followed by centrifugation at 2000 x *g* for 10 min to obtain the supernatant (S1). 250µL of S1 was incubated with 600µL of TBA (0.6%), 600µL of phosphoric acid (1%) and 50µl of distillated water for 2 h at 95 °C and the absorbance was measured spectrophotometrically at 532 nm. A curve using malondialdehyde (MDA) as standard was constructed in order to express the results in nmol MDA/mg protein.

2.5.6Total thiol and non-protein thiol (NPSH) level determination

Liver and kidney thiol (SH) levels were determined in S1 as previously described by Ellman [36]. For non-protein thiol determination, the protein fraction contained in S1 was precipitated using TCA 4% in 1/1 proportion, followed by centrifugation at 2000 x *g* for 10 min, in order to obtain the S2 that was used for analysis. The absorbance was determined at 412 nm. A curve using glutathione as standard was constructed in order to calculate the SH in the tissue samples. The thiol levels were expressed as nmol SH/mg protein.

2.6 Hg and Se content determination

Hg content was determined by inductively coupled plasma atomic emission spectrometry (ICPE-9000; Shimadzu Scientific Instruments). Samples of kidney, liver and blood, 0.5 g, 1.0 g and 0.5 ml, respectively, were collected and placed in vials and frozen at -20 °C for subsequent digestion. The samples were digested in distilled HNO_3 as described by Ineu et al. [37] and ultimately samples were diluted in deionized water and Hg determination was carried out using ICPE-9000. For Se determination, samples were prepared correspondingly to Hg. For the determination of this element, a Hydride Vapor Generator system (HVG-1; Shimadzu Scientific Instruments) was also necessary. The analytical standard Hg and Se (Merck®) was used to make the standard curve (0, 50, 150, 300, 600, 1200 ppb) (*r=0.99323*) and (0, 0.05, 0.1, 0.5, 1, 5, 10, 15 and 20 ppm) (*r=0.99396*) respectively.

2.7 Protein determination

Protein content was determined by Bradford [38], using bovine serum albumin as a standard.

2.8 Statistical analysis

Results were analyzed by one-way ANOVA of variance followed by Duncan`s multiple range test when appropriate. Results were considered significant when $p \leq 0.05$. Groups statistically equal are accompanied by the same letters, and groups statistically different are accompanied by different letters. In some cases, two groups were compared by Student *t* test ($p \leq 0.05$) and the difference was represented by (*).

3. Results

3.1 Biochemistry assays:

3.1.1 δ-ALA-D activity

Renal, hepatic and blood δ-ALA-D activity are shown in Fig.1. The treatments did not cause any alteration in the enzyme activity from kidney (Fig. 1A) and liver (Fig. 1B), but significantly decreased blood (Fig.1C) δ-ALA-D activity [ANOVA, $F_{(3,45)}=3.748$; $p = 0.02$)]. Hg-exposure inhibited the blood δ-ALA-D activity, and $(PhSe)_2$ pre-exposure partially prevented this effect (Duncan $p \leq 0.05$). $(PhSe)_2$ alone did not affect the δ-ALA-D activity from all tissues studied.

3.1.2 Serum AST and ALT activities

Hepatic toxicity biomarker, AST and ALT activities, are shown in Fig. 2. AST activity was not affected by treatment (Fig. 2A). The lower ALT activity (Fig. 2B) observed in those animals treated with the $(PhSe)_2$ and/or $HgCl_2$ was not statistically significant [$F_{(3,24)}=2.449$; $p = 0.08$]. However, when the Hg group was compared with control by Student *t* test, a significant ALT inhibition was observed [$t_{(12)}=2.428$; $p=0.03$].

3.1.3 Serum urea and creatinine levels

Serum urea and creatinine levels are shown in Fig.3. Urea levels were not altered by the $HgCl_2$ or $(PhSe)_2$ treatment (Fig. 3A). An alteration in creatinine levels (Fig 3B) was observed [$F_{(3,22)}$=3.931; p = 0.02]; $HgCl_2$ increased creatinine levels when compared to the control group, which was prevented by $(PhSe)_2$ pre-treatment (Duncan $p \leq 0.05$). $(PhSe)_2$ *per se* did not affect creatinine levels.

3.1.4 TBARS levels

Renal, hepatic and blood TBARS levels are shown in Fig.4.When the Hg group was compared with control, Student *t* test revealed a decrease in kidney (Fig. 4A) TBARS levels in Hg exposed mice [$t_{(20)}$= 2.657; p=0.02].

Hepatic (Fig. 4B) and blood (Fig. 4C) TBARS levels were not affected by the treatments.

3.1.5 Determination of Total Thiols and Non-protein thiols (NPSH)

The levels of total thiols and NPSH in kidney and liver are shown in Fig. 5 and 6, respectively. Treatments did not cause any alteration in total thiols in either studied tissues. However, a significant increase in kidney NPSH levels (Fig. 6A) was observed in Hg group [$F_{(3,38)}$=2.765; p = 0.05]. Animals that received only $(PhSe)_2$ also demonstrated an increase in kidney NPSH levels; however, this alteration was not significant. Even the group that received $(PhSe)_2$ plus $HgCl_2$ showed an increase in kidney NPSH levels, similar to what was presented by the $(PhSe)_2$ group. The liver NPSH levels (Fig. 6B) were not altered by treatments.

3.2 Hg and Se content determination:

3.2.1 Hg content

The content of Hg in kidney, liver and blood are shown in Table 1. Both Hg exposed groups (Hg group and Se-Hg group) presented an increase of Hg content in kidney [$F_{(3,8)}$=50.413; p = 0.001)], liver [$F_{(3,8)}$=5.814;p = 0.02)] and blood [$F_{(3,8)}$=10.562; p = 0.004)]. Moreover, the Se-Hg group presented the kidney Hg level higher and blood Hg level lower than Hg group (Duncan $p \leq 0.05$). $(PhSe)_2$, pre-treatment did not alter the Hg accumulation in liver.

3.2.2 Se content

The content of Se in kidney, liver and blood are shown in Table 1. The Se content in kidney and liver was not altered by the treatments. On the other hand, a significant decrease in blood Se content was observed [$F_{(3,8)}$=6,162; p = 0.01)]. The animals exposed to Hg or $(PhSe)_2$ or both showed a slight but significant decrease in blood Se content when compared to the control group (Duncan $p \leq 0.05$).

4. Discussion

This study was conducted in order to evaluate the effects of $(PhSe)_2$ pre-treatment against the inorganic Hg exposition. In order for this to be possible, hepatic and renal toxicity parameters were analyzed, as well as oxidative parameters and Hg and Se content were determined. The current research attempts to simulate a daily ingestion of selenium in order to prevent the damages caused by exposure to Hg. $HgCl_2$ subcutaneously administration is well described in the literature and was used in our study in order to evaluate the mechanism involved in Hg exposure and to obtain classical characteristics of Hg poisoning. The δ-ALA-D is a sulfhydryl enzyme present in different tissues, *e.g.* renal, hepatic and blood. Various

heavy metals, such as Hg, have affinity to sulfhydryl groups and this affinity is an important contributor to its toxicity [39]. Since Hg is a non-essential metal, and several studies have reported its toxicity, the enzyme δ-ALA-D can be an important biomarker of toxic effects of this metal [14,17,40,41]. In our study, renal and hepatic δ-ALA-D activity was unaltered by $HgCl_2$ exposure, differently from blood δ-ALA-D activity which was inhibited by $HgCl_2$. Blood δ-ALA-D inhibition of 60% was previously demonstrated in a study where young ratsreceived $HgCl_2$ 5.0 mg/kg during five consecutive days [40], the same dose used in our study. Indeed, in our study, pre-treatment with $(PhSe)_2$ partially prevented the δ-ALA-D inhibition in blood tissue. $(PhSe)_2$ *per se* did not cause any δ-ALA-D activity alterations.

Serum ALT and AST activities were determined in order to evaluate hepatic toxicity. Serum ALT activity inhibition was observed in those animals that received only Hg. These results are all in agreement with previous studies presented by our group [17,41]. However, this effect cannot be characterized as hepatotoxicity, once the cellular lesion in the liver is characterized by presenting an increase in serum ALT activity [42]. This inhibition probably may be due to direct interaction between $HgCl_2$ and sulfhydryl groups present in the ALT structure. Results of the inhibitory effect revealed that sulfhydryl groups are directly involved in their enzymatic activity [43]. In our study, AST activity was not altered.

Serum urea and creatinine levels are used as an indicator of impaired renal function [44]. It is well described that nephrotoxic effects of Hg lead to renal dysfunction and consequently increase in serum urea and creatinine levels [12,41]. In this experiment urea levels were not altered in any group, only creatinine levels were increased in the Hg group. Urea represents the principal product of protein catabolism and, due to a decrease in the glomerular filtration rate, their levels are increased in the bloodstream [44,45]. According to Gilbert et al.[46], increase in urea levels occurs only after parenchyma tissue injury. On the other hand, creatinine levels are a significant indicator during the first phases of kidney

disease [47]. This increase, only in creatinine levels, may represent a possible initial process of renal damage which was completely prevented by the $(PhSe)_2$. This kind of protection against the increase in creatinine levels can be attributed to the antioxidants $(PhSe)_2$ property [28], since this compound proves to be efficient in preventing oxidative damage by cadmium in different tissues [26,30]. On the other hand, the exact mechanism involved in this protection is not well understood.

Hg exposure may promote the formation of reactive oxygen species (ROS) and, consequently, induce lipid peroxidation in different tissues as renal and hepatic that can be detected by increase of TBARS levels [48-51]. Our results differ from those presented by Brandão et al. [52] who reported an increase in renal TBARS levels of mice exposed to 1.0mg/kg of $HgCl_2$ for two weeks. The decrease of TBARS levels in the kidney observed in our study may be due to an adaptive response against Hg exposure (discussed further below).

One further oxidative parameter analyzed was NPSH levels. It was detected an increase of NPSH levels in the kidneys of animals exposed to $HgCl_2$ which was partially prevented by $(PhSe)_2$. Our results are in agreement with the results reported by Brandão et al.[53]. GSH is the most abundant endogenous NPSH present in all mammalian tissue which plays an important role in protecting cells [11,54]. Inorganic Hg, in low or nontoxic doses, may lead to a renal GSH level increase, probably due to the induction of GSH synthesis [55]. The increase in NPSH levels observed in our study may be associated with an increase of GSH levels. In agreement with this, the renal lipid peroxidation decrease, previously mentioned, may be due to an increase in GSH which plays a crucial role in protecting the cells against lipid peroxidation and has antioxidant activity as intracellular free radical scavenger [11, 56].

Animals exposed to Hg demonstrated an Hg deposition in kidney about 3 times more than liver. As already acknowledged, the kidney is the primary target organ where inorganic

Hg accumulates, and expresses its toxicity [55]. These results are in agreement with previous research performed by our group [14,17,18,57]. The animals that received $(PhSe)_2$ pre-treatment (Se-Hg group) demonstrated an Hg retention in kidney 30% more than those animals that received only Hg (Hg group). This may occur due to $(PhSe)_2$ capacity to increase GSH levels, as well as a sulfhydryl containing molecule [58] which may bind to Hg and increase its retention [55]. In accordance with this, a study performed by Tanaka et al. [59] demonstrated that renal $HgCl_2$ accumulation decreases after GSH depletion. Another possible mechanism regarding kidney Hg is that the accumulation may be enhanced in metallothionein (MT) content. In fact, Brandão et al. [53] demonstrated that $(PhSe)_2$ may increase the MT content, since this protein is a cysteine-rich protein which may bind to Hg and reduce its toxicity. Similar to GSH, the intracellular content of MT may be altered by several substances, leading to alter Hg toxicity and intracellular distribution [55]. Although this was observed at the same time that occurred an increase in renal Hg retention, a decrease in blood Hg levels was also observed in those from animals pre-treated with $(PhSe)_2$, thus this maydenote a capacity of $(PhSe)_2$ in Hg detoxification.

In this research, we observed that $HgCl_2$ exposition leads to inhibition in blood δ-ALA-D and serum ALT activity, which was partially prevented by $(PhSe)_2$ treatment. The involved mechanism in this protection may be related to the $(PhSe)_2$ ability to remove partial Hg content in blood and kidney to be eliminated, thus, a smaller quantity of Hg is available to react with the sulfhydryl groups and express its toxicity. We believe that this protection is related to the $(PhSe)_2$ antioxidant property and its ability to increase GSH levels. Additionally, we speculate that a MT/GSH-Hg complex is formed when Hg is absorbed, and this complex is unable to react with the cellular components. Corroborating with this hypothesis, total protection against increase in creatinine levels in the Se-Hg group was observed, although this

group displayed higher levels of Hg in the kidney. This may indicate that MT/GSH-Hg complex is an inert complex.

Selenium plays an important role in the antioxidant defense of the organism, and there is an increasing search for understanding its biological role, in particular its use as therapeutic agent [59]. Studies have shown that Se and Hg can interact in the body of mammals reducing Hg toxicity [60]. Thus, Se can be a promissory agent in the prevention of Hg to exposed populations.

Accordingly, $(PhSe)_2$ proves to be effective in protecting against Hg toxicity in order to improve the antioxidant system, and once Hg is attached to antioxidants agents, such as MT and GSH, it is unable to react with sulfhydryl groups in biomolecules. However, further investigations are necessary to elucidate the precise mechanism regarding this protection.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Legends:

Figure 1: Kidney (A), Liver (B) and Blood (C) δ-ALA-D activity. Male Swiss albino mice were exposed to $(PhSe)_2$ (5.0 mg/kg/day; v.o.) or oilfor five days and subsequently exposed another five days to $HgCl_2$ (5.0 mg/kg/day, s.c.) or saline and killed 24 hours after the last $HgCl_2$ administration. The results are expressed as mean ± S.E.M. Duncan's multiple range test: groups statistically equals are accompanied of the same letters, and groups statistically different are accompanied by different letters. ($p \leq 0.05$; n = 7-12).

Figure 2: Serum AST (A) and ALT (B) activity from mice treated as described in Fig. 1. The results are expressed as mean ± S.E.M. ($p \leq 0.05$; n = 7). (*) differ from control group: Student *t* test.

Figure 3: Serum Urea (A) and Creatinine (B) levels from mice treated as described in Fig. 1. The results are expressed as mean ± S.E.M. Duncan's multiple range test: groups statistically equals are accompanied by the same letters, and groups statistically different are accompanied by different letters. ($p \leq 0.05$; n = 6-8).

Figure 4: Kidney (A), Liver (B) and Blood (C) TBARS levels from mice treated as described in Fig. 1. The results are expressed as mean ± S.E.M. ($p \leq 0.05$; n = 10-13). (*) differ from control group: Student *t* test.

Figure 5: Kidney (A) and liver (B) total thiol levels from mice treated as described in Fig. 1. The results are expressed as mean ± S.E.M. (n = 10-12).

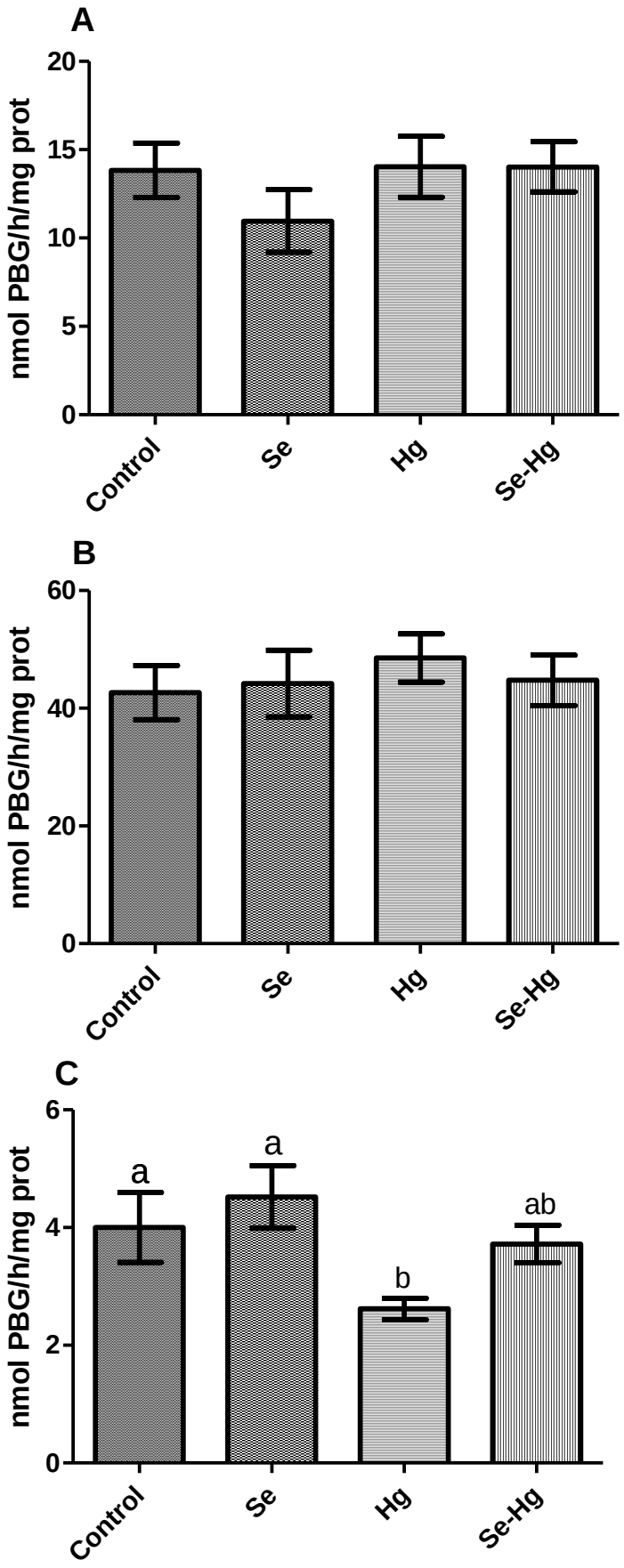
Figure 6: Kidney (A) and Liver (B) NPSH levels from mice treated as described in Fig. 1. The results are expressed as mean ± S.E.M. Duncan's multiple range test: Groups statistically equal are accompanied by the same letters, and groups statistically different are accompanied by different letters. ($p \leq 0.05$; n = 10-12).

Table 1. Hg and Se levels in liver, kidney and blood from mice exposed to $(Ph_2Se)_2$ (5 mg/kg/day) or saline for five consecutive days and exposed to $HgCl_2$ (5 mg/kg/day) or saline on the five subsequent days.

	Liver	**Kidney**	**Blood**
Groups	**Hg levels (µg Hg/g tissue)**		
Control	nd[a]	1.70±0.24[a]	0.20±0.01[a]
Se	nd[a]	1.12±0.17[a]	0.21±0.01[a]
Hg	4.65±1.78[b]	10.95±0.79[b]	0.52±0.08[b]
Se-Hg	4.56±1.30[b]	14.54±1.69[c]	0.36±0.05[c]
Groups	**Se levels (µg Se/ g tissue)**		
Control	2.83±0.07	2.63±0.09	0.28±0.01[a]
Se	2.53±0.08	2.43±0.04	0.24±0.02[b]
Hg	2.59±0.11	2.57±0.03	0.23±0.01[b]
Se-Hg	2.77±0.02	2.65±0.08	0.24±0.00[b]

Data are expressed as mean ± S.E.M. (n = 3) and the values followed by different letters in the same column are statistically different ($P<0.05$). The samples whose mercury concentrations were below the detectable limit (non detected, nd) of the technique were considered, for statistical analysis, as containing 0.02 µg of metal/g of tissue, which is the minimum measurable quantity.

Figure 1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Control
Se
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B
nmol PBG/h/mg prot
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20
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Control
Se
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Se
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Figure 2

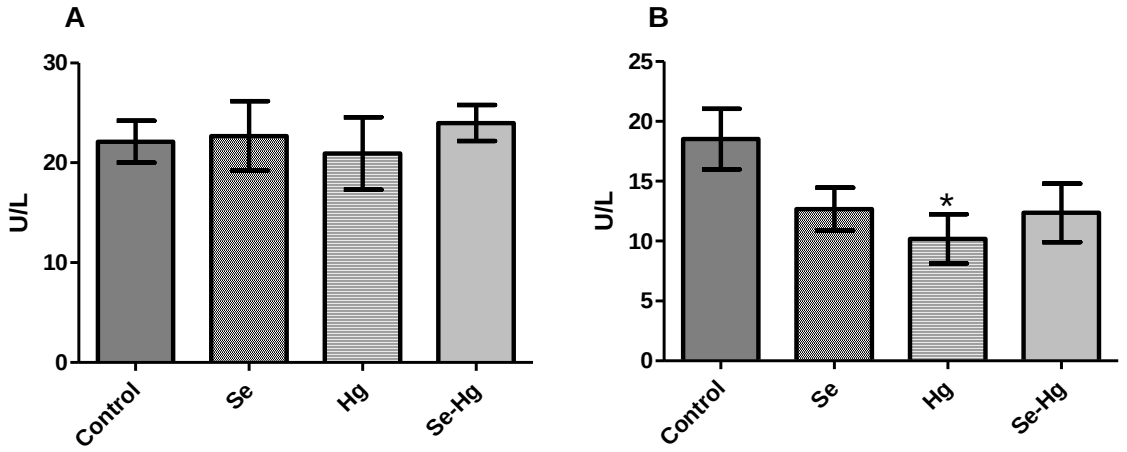


Figure 3

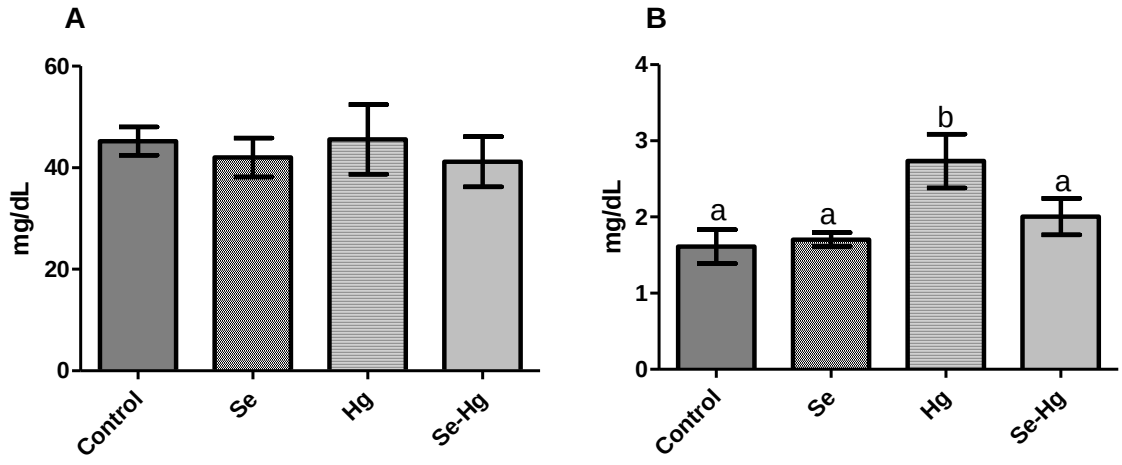
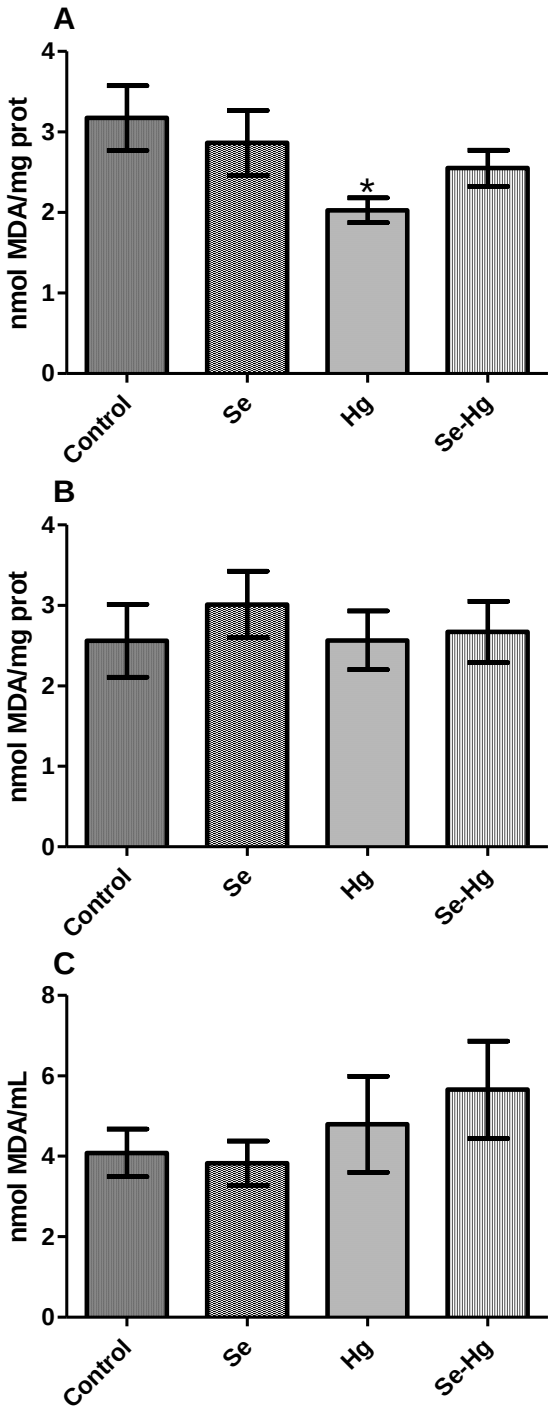


Figure 4


A
nmol MDA/mg prot
4
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Control
Se
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Se-Hg

Figure 5

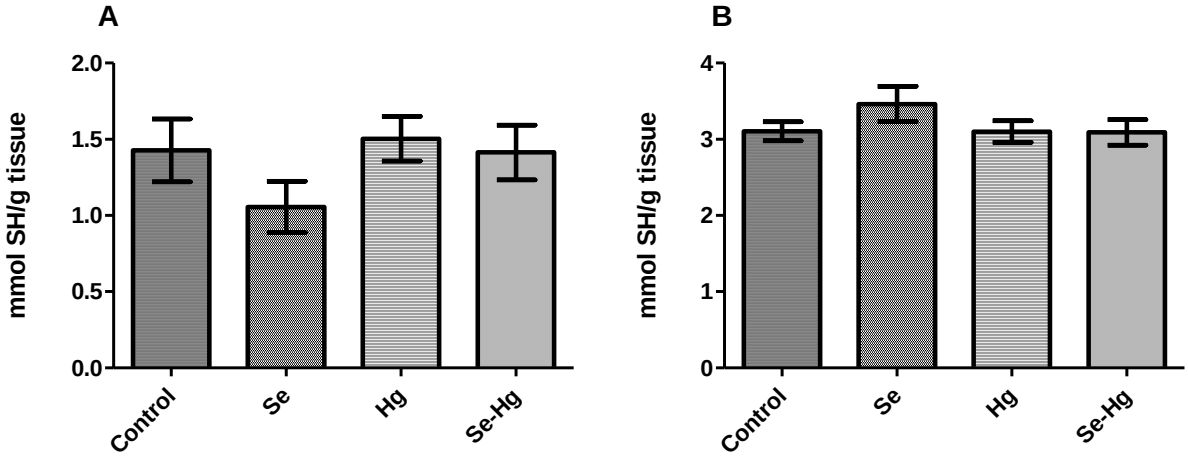
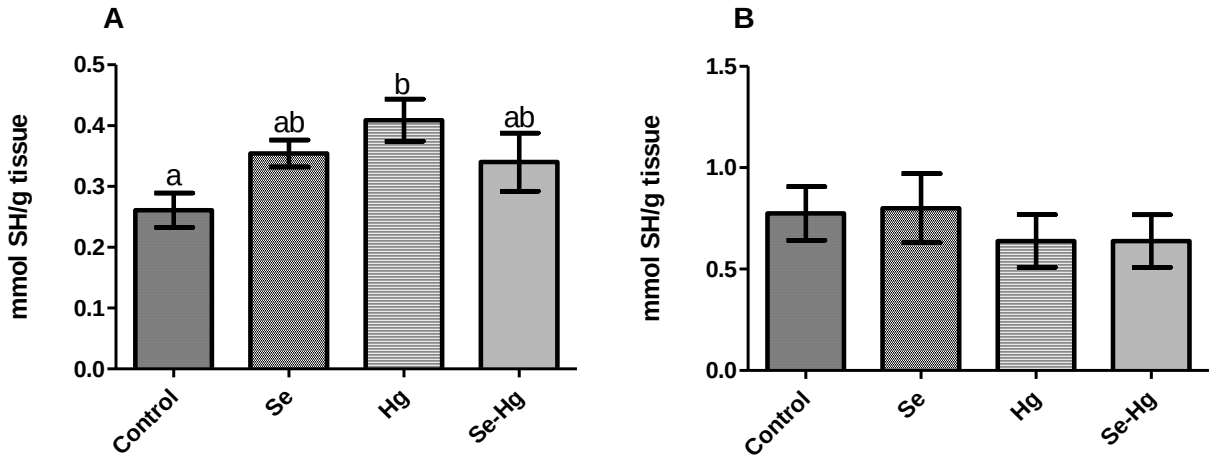

A
mmol SH/g tissue
2.0
1.5
1.0
0.5
0.0
Control
Se
Hg
Se-Hg
B
mmol SH/g tissue
4
3
2
1
0
Control
Se
Hg
Se-Hg

Figure 6


A
mmol SH/g tissue
0.5
0.4
0.3
0.2
0.1
0.0
a
ab
b
ab
Control
Se
Hg
Se-Hg
B
mmol SH/g tissue
1.5
1.0
0.5
0.0
Control
Se
Hg
Se-Hg

4. CONCLUSÕES

A partir dos resultados deste estudo, é possível sugerir que o disseleneto de difenila possa ser um agente com atividade protetora contra os efeitos do mercúrio tendo em vista que:

- ✓ Apesar da exposição ao Hg não causar a inibição da enzima δ-ALA-D nos tecidos renal e hepático, foi observado uma maior sensibilidade da enzima sanguínea ao Hg, o que foi protegido parcialmente pelo $(PhSe)_2$.
- ✓ A exposição ao $HgCl_2$ causou um aumento nos níveis séricos de creatinina mas não de ureia, o que pode representar o início de um dano renal. O pré-tratamento com $(PhSe)_2$ preveniu totalmente esse efeito causado pelo $HgCl_2$.
- ✓ Os animais expostos ao $HgCl_2$ não evidenciaram dano hepático, pois não foram observadas alterações na atividade da enzima AST sérica. Foi observada apenas a inibição da atividade da enzima ALT sérica, o que provavelmente é devido a interação do Hg aos grupamentos SH presentes na enzima.
- ✓ A exposição ao $HgCl_2$ aumentou os níveis de tiois não protéicos, provavelmente devido a uma resposta adaptativa consequente da exposição ao metal, o que foi parcialmente protegido pelo $(PhSe)_2$. Contudo uma diminuição, inesperada, nos níveis de TBARS foi observada nos animais expostos ao $HgCl_2$.
- ✓ A exposição ao $(PhSe)_2$, $HgCl_2$ ou ambos induziu uma pequena diminuição nos níveis de Se no sangue. O rim foi o órgão que apresentou maior retenção de Hg, sendo que o pré-tratamento com $(PhSe)_2$ causou uma retenção ainda maior de Hg neste órgão, ao mesmo tempo em que ocorreu uma diminuição deste metal no tecido sanguíneo. Esse efeito do $(PhSe)_2$ na distribuição do Hg entre os tecidos, pode ter contribuído para a proteção parcial na atividade das enzimas ALT e δ-ALA-D sanguínea.

5. PERSPECTIVAS

Considerando os resultados apresentados, faz-se necessário a determinação de outros parâmetros envolvidos na toxicidade do mercúrio. Considerando que o mercúrio acumula-se mais nos rins para ser eliminado, faz-se necessário a análise de outros materiais biológicos, tais como urina e fezes. Sendo assim, mais estudos são necessários para definir o mecanismo pelo qual o disseleneto de difenila exerce atividade protetora contra os efeitos tóxicos do mercúrio no modelo testado.

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