UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS NATURAIS E EXATAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA
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EFEITO DO DISSELENETO DE DIFENILA SOBRE A TOXICIDADE INDUZIDA POR CLORETO DE MERCÚRIO EM CAMUNDONGOS

TESE DE DOUTORADO

Ricardo Brandão

Santa Maria, RS, Brasil
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EFEITO DO DISSELENETO DE DIFENILA SOBRE A TOXICIDADE INDUZIDA POR CLORETO DE MERCÚRIO EM CAMUNDONGOS

por

Ricardo Brandão

Tese 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
Doutor em Bioquímica Toxicológica.

Orientadora: Prof^a. Dr^a. Cristina Wayne Nogueira
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A Comissão Examinadora, abaixo assinada, aprova a Tese de Doutorado

EFEITO DO DISSELENETO DE DIFENILA SOBRE A TOXICIDADE INDUZIDA POR CLORETO DE MERCÚRIO EM CAMUNDONGOS

Elaborada por **Ricardo Brandão** como requisito parcial para a
obtenção do grau de **Doutor em Bioquímica Toxicológica**

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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Bioquímica Toxicológica
Universidade Federal de Santa Maria, RS, Brasil

EFEITO DO DISSELENETO DE DIFENILA SOBRE A TOXICIDADE INDUZIDA POR CLORETO DE MERCÚRIO EM CAMUNDONGOS
AUTOR: Ricardo Brandão
ORIENTADORA: Cristina Wayne Nogueira
DATA E LOCAL DA DEFESA: Santa Maria, dezembro de 2008.

O mercúrio (Hg) é um elemento ainda sem função fisiológica no organismo humano, sendo tóxico aos seres vivos. Este metal possui ampla aplicação na indústria sendo, portanto, bastante importante na exposição ocupacional e ambiental. A toxicidade do mercúrio depende da forma deste metal e pode afetar inúmeros órgãos, tais como o cérebro, os rins e o fígado e, ainda, causar alterações hematológicas e imunológicas. O estresse oxidativo parece estar envolvido na toxicidade induzida pelo mercúrio, uma vez que este metal pode causar um aumento na produção de espécies reativas de oxigênio (EROs) e distúrbios nos sistemas de defesa antioxidante enzimáticos e não-enzimáticos. Desta forma, além das terapias convencionais por meio de agentes quelantes, terapias utilizando agentes antioxidantes são testadas na tentativa de reduzir os efeitos tóxicos deste metal. Considerando que o composto disseleneto de difenila $(PhSe)_2$ possui inúmeras propriedades farmacológicas, dentre as quais destaca-se a sua ação antioxidante, o nosso objetivo foi verificar o efeito deste composto em diferentes modelos de exposição ao cloreto de mercúrio $(HgCl_2)$ em camundongos. As utilizações de outro agente antioxidante, a N-acetilcisteína (NAC), e de um agente quelante de referência, o ácido 2,3-dimercapto-1-propanosulfônico (DMPS), também foram avaliadas. Os resultados obtidos demonstraram que quando o $HgCl_2$ foi administrado de forma aguda e a utilização dos agentes terapêuticos testados ocorreu de forma concomitante, efeitos tóxicos decorrentes destas interações foram observados. De fato, a administração de NAC e DMPS, em animais expostos ao $HgCl_2$, causou toxicidade renal nos camundongos, o que foi evidenciado através de um aumento nos níveis de uréia e creatinina e através da redução na atividade da enzima Na^+, K^+-ATPase renal. Esta toxicidade foi devida a uma possível formação de complexos tóxicos entre o metal e estes agentes. A administração de $(PhSe)_2$ causou 100% de morte nos animais expostos ao $HgCl_2$. Os efeitos tóxicos decorrentes desta associação afetam o tecido hepático e, principalmente, o tecido renal. O dano hepático foi caracterizado pelo aumento nos níveis de peroxidação lipídica e redução na atividade da enzima catalase nos animais do grupo $HgCl_2$ + $(PhSe)_2$. O dano renal foi caracterizado através de marcadores bioquímicos no plasma e na urina dos camundongos. Além disso, os camundongos expostos a associação entre o $HgCl_2$ e o $(PhSe)_2$ apresentaram inibições na atividade das enzimas glutationa S-transferase (GST) e Na^+, K^+-ATPase renal. O dano oxidativo no tecido renal foi evidenciado através do aumento nos níveis de peroxidação lipídica e aumento na concentração de ácido ascórbico nos camundongos expostos ao $HgCl_2$ e ao $(PhSe)_2$, de forma concomitante. Elevados valores de hemoglobina e hematócrito também foram observados nos

camundongos do grupo $HgCl_2$ + $(PhSe)_2$ e o dano renal parece estar envolvido neste efeito. A formação de um complexo entre o $HgCl_2$ e o $(PhSe)_2$, o qual apresenta atividades pró-oxidantes, é a hipótese mais provável para explicar esta toxicidade. Foi observado também que a terapia preventiva com o $(PhSe)_2$ foi efetiva em proteger contra os danos nos sistemas hematológico e imunológico induzidos de forma subcrônica pelo $HgCl_2$. De fato, a exposição ao $HgCl_2$ causou anemia nos camundongos, o que foi observado através da redução nos níveis de hemoglobina, contagem de eritrócitos e no hematócrito. Além disso, os níveis de leucócitos e plaquetas também foram reduzidos pela exposição ao metal. As alterações imunológicas foram evidenciadas pelo aumento nos níveis de imunoglobulinas. Todas estas alterações, hematológicas e imunológicas, foram reduzidas pelo pré-tratamento com o $(PhSe)_2$. A ação antioxidante deste composto de selênio parece estar envolvida neste mecanismo de proteção, bem como a formação de um complexo ternário inerte entre o mercúrio, o selênio e a selenoproteína P. O $(PhSe)_2$ também foi tão efetivo quanto o DMPS em reverter os danos renal e hematológico observados após a exposição subcrônica ao $HgCl_2$. As alterações hematológicas (diminuição nos níveis de eritrócitos, leucócitos e plaquetas) e as alterações no tecido renal, observadas através do aumento nos níveis de uréia, creatinina e ácido úrico plasmáticos e através da peroxidação lipídica renal, induzidos pela exposição ao $HgCl_2$, foram revertidas pelas administrações individuais de $(PhSe)_2$ e DMPS. Entretanto, a utilização do $(PhSe)_2$ de forma associada ao DMPS não apresentou bons resultados, uma vez que as administrações individuais destes dois agentes foram mais eficazes do que a administração combinada. Com base nos resultados obtidos, nós podemos concluir que a utilização do $(PhSe)_2$ em intoxicações pelo $HgCl_2$ deve ser ainda mais estudada, já que, dependendo do modelo experimental utilizado, os resultados podem ser benéficos ou pode haver uma potencialização dos efeitos tóxicos do mercúrio.

Palavras-chave: selênio, mercúrio, disseleneto de difenila, N-acetilcisteína, DMPS, dano renal.

ABSTRACT

Thesis of Doctor's Degree
Federal University of Santa Maria, RS, Brazil

EFFECT OF DIPHENYL DISELENIDE ON TOXICITY INDUCED BY MERCURIC CHLORIDE IN MICE
AUTHOR: Ricardo Brandão
ADVISOR: Cristina Wayne Nogueira
DATE AND PLACE OF THE DEFENSE: Santa Maria, 2008

Mercury is a metal without physiological functions in human body and is toxic to human beings. This metal has many applications in industry and, therefore it is very important in occupational and environmental exposure. The toxicity of mercury depends on the form of the metal and can affect several organs, such as brain, kidney and liver. In addition, mercury can cause alteration in hematological and immunological systems. The oxidative stress seems to be involved in toxicity induced by mercury, since this metal may cause an increase in the production of reactive oxygen species (ROS) and disturbances in enzymatic and non-enzymatic antioxidant defense systems. Thus, in addition to conventional therapies using chelating agents, therapies using antioxidants are tested in an attempt to reduce the toxic effects of this metal. Since diphenyl diselenide $(PhSe)_2$ has several pharmacological properties, including antioxidant action, our goal was to verify the effect of this compound in different models of exposure to mercuric chloride $(HgCl_2)$ in mice. The use of another antioxidant agent, N-acetylcysteine (NAC), and a chelating agent of reference, 2,3-dimercapto-1-propanosulfonato (DMPS), were also evaluated. The results showed that the concomitant and acute exposure to $HgCl_2$ and therapeutic agents tested presented toxic effects. In fact, the administration of DMPS and NAC, in animals exposed to $HgCl_2$, caused renal toxicity in mice, which was evidenced by an increase in the urea and creatinine levels and by reduction on renal Na^+, K^+-ATPase activity. This can be explained by a possible formation of complexes between the metal and these agents. The administration of $(PhSe)_2$ caused 100% of death in animals exposed to $HgCl_2$. The toxic effects of $HgCl_2 + (PhSe)_2$ association affects the hepatic tissue and especially the renal tissue. Hepatic damage was characterized by an increase in the lipid peroxidation levels and reduction in catalase activity from animals of $HgCl_2 + (PhSe)_2$ group. Renal damage was characterized by biochemical markers in plasma and urine of mice. Moreover, mice exposed to the association between $HgCl_2$ and $(PhSe)_2$ showed inhibitions in renal glutathione S-transferase (GST) and Na^+, K^+-ATPase activities. The oxidative damage in renal tissue was evidenced by increase in the lipid peroxidation levels and increase in ascorbic acid concentration in mice exposed to $HgCl_2 + (PhSe)_2$ group. Increased levels of hemoglobin and hematocrit were also observed in mice of $HgCl_2 + (PhSe)_2$ group and renal damage seems to be involved in this effect. The formation of a complex between $HgCl_2$ and $(PhSe)_2$, which displays pro-oxidant activity, is the most probably hypothesis to explain this toxicity. We observed also that the preventive therapy with $(PhSe)_2$ was effective in protecting against immunological and hematological alterations induced by subchronic $HgCl_2$ exposure. In fact, exposure to

$HgCl_2$ caused anemia in mice, which was observed by reducing in the hemoglobin, erythrocytes and hematocrit levels. Moreover, levels of leukocytes and platelets were also reduced by exposure to $HgCl_2$. The immunological changes were evidenced by increase in immunoglobulins levels. All these changes, hematological and immunological, were reduced by $(PhSe)_2$ pre-treatment. The antioxidant activity of this selenium compound seems to be involved in this mechanism of protection, as well as the formation of a inactive ternary complex between mercury, selenium and selenoprotein P. $(PhSe)_2$ also presented similar effects when compared to DMPS in restored renal and hematological damage observed after subchronic exposure to $HgCl_2$. The hematological changes (decrease in erythrocytes, leukocytes and platelets levels) and changes in renal tissue, observed by increase in the plasmatic urea, creatinine, and uric acid levels and renal lipid peroxidation, induced by exposure to $HgCl_2$, were reversed by $(PhSe)_2$ and DMPS, individually administered. However, the combined use of $(PhSe)_2$ and DMPS did not present good results, since the individual therapies with these two agents were more effective than the combined administration. Based on these results, we can conclude that the use of $(PhSe)_2$ against the $HgCl_2$ toxicity should be further studied, since, depending on the experimental model, the results can be beneficial or there may be a potentiation of the toxic effects of mercury.

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ESQUEMAS

LISTA DE ABREVIATURAS

δ-ALA – ácido 5'-aminolevulínico ou ácido delta-aminolevulínico

δ-ALA-D - delta-aminolevulinato desidratase ou porfobilinogênio sintase

ALT – alanina aminotransferase

AMPc – 3'-5' adenosina monofosfato cíclico

ANOVA – análise de variância

AST – aspartato aminotransferase

ATP –adenosina tri-fosfato

BAL – 2,3-dimercaptopropanol, dimercaprol

CAT – catalase

CHCM – concentração de hemoglobina corpuscular média

Cys – cisteína

DL50 - dose letal para 50 % dos animais

DMPS – ácido 2,3-dimercaptopropano 1-sulfônico

DMSA – ácido meso-2,3-dimercaptosuccínico

DMSO – dimetilsulfóxido

DNA – ácido desoxirribonucléico

EROs – espécies reativas de oxigênio

GPx – glutationa peroxidase

GR – glutationa redutase

GSH – glutationa reduzida

GST – glutationa S-transferase

HCM – hemoglobina corpuscular média

Hg^0 - mercúrio elementar

H_2O_2 - peróxido de hidrogênio

i.p. – intraperitoneal

IgG – imunoglobulina G

IgM – imunoglobulina M

LDH – lactato desidrogenase

MDA - malondialdeído

MT- metalotioneina

NAC – N-acetilcisteína

NPSH – tióis não-protéicos

$O_2^{\bullet-}$ - ânion superóxido

$^1O_2$ - oxigênio singlete

$OH^{\bullet}$ - radical hidroxil

PBG - porfobilinogênio

$(PhSe)_2$ – disseleneto de difenila

RDW – Red Cell Distribution Width

RNA – ácido ribonucléico

S.D – standard deviation

S1 – sobrenadante

S.E.M – standard error of the mean

-SH – grupo sulfidrila

SNC – Sistema Nervoso Central

SOD – superóxido dismutase

s.c. – subcutânea

TBARS – espécies reativas ao ácido tiobarbitúrico

VCM – volume corpuscular médio

SUMÁRIO

1. INTRODUÇÃO

Os metais pesados são alguns dos principais contaminantes encontrados no meio ambiente. Estes metais têm amplo emprego industrial, constituindo uma das principais formas de intoxicação ocupacional e ambiental (Salgado, 1996). Dentre os metais de maior preocupação estão o chumbo, o mercúrio, o arsênio e o cádmio (Klaassen, 1996). O mecanismo molecular envolvido na toxicidade destes metais está relacionado com a produção de estresse oxidativo, seja através da redução de defesas antioxidantes ou pela geração de espécies reativas de oxigênio (EROs) (Hussain et al., 1999; Wang et al., 2004).

O mercúrio (Hg) é um elemento mineral metálico ainda sem função fisiológica no organismo humano, sendo tóxico aos seres vivos (Esquerda et al.,1989). Este metal possui ampla aplicação na indústria, podendo, dessa forma, atingir trabalhadores e a população em geral (Boischio e Henshel, 1996; Klaassen, 1996). Sabe-se que o mercúrio pode causar diversos prejuízos ao organismo, afetando principalmente os sistemas nervoso, gastrintestinal, renal e hepático (Larini et al., 1997). O estresse oxidativo parece estar envolvido na toxicidade induzida pelo mercúrio, uma vez que este metal pode causar um aumento na produção de EROs, ocasionando danos em lipídios, proteínas e no ácido desoxirribonucléico (DNA) (Goering et al., 2002). Além disso, os sistemas de defesa antioxidante enzimáticos e não-enzimáticos podem ser reduzidos pela exposição ao mercúrio (Hussain et al., 1999).

A forma mais efetiva de tratamento contra intoxicações por metais consiste na utilização de agentes quelantes (Vamnes et al., 2003). São exemplos de agentes quelantes, o ácido 2,3-dimercapto-1-propanosulfônico (DMPS, Dimaval®) e o ácido *meso*-2,3-dimercaptosuccínico (DMSA, Succimer®), os quais são menos tóxicos e, possivelmente, mais efetivos do que outros agentes quelantes no tratamento destas intoxicações (Faria, 2003).

Acredita-se que, além da terapia convencional por meio de agentes quelantes, a terapia utilizando agentes antioxidantes possa ser efetiva em proteger contra os danos causados pelo mercúrio. São diversos os antioxidantes utilizados na tentativa de reverter os danos teciduais devido ao estresse oxidativo induzido pelo mercúrio. A N-

acetilcisteína (NAC), a qual contém grupos tióis em sua estrutura, é um antioxidante bastante utilizado em condições de estresse oxidativo (Cortijo et al., 2001). Além disso, o nosso grupo de pesquisa tem demonstrado que o composto disseleneto de difenila $(PhSe)_2$, um composto orgânico de selênio, possui inúmeras propriedades farmacológicas (Nogueira et al., 2003a; 2004; Borges et al., 2005a; 2006; Barbosa et al., 2006; Savegnago et al., 2006; 2007), dentre as quais se destaca a sua ação antioxidante (Meotti et al., 2004). Considerando os aspectos acima mencionados, é de grande relevância a avaliação do efeito do $(PhSe)_2$, bem como de outros agentes terapêuticos, frente à toxicidade induzida pelo cloreto de mercúrio $(HgCl_2)$ em camundongos.

2. OBJETIVOS

Objetivo Geral

Os efeitos tóxicos provocados por metais pesados, como o mercúrio, têm recebido bastante atenção dos pesquisadores. Muitos estudos têm sido realizados com o objetivo de verificar as alterações causadas por este metal no organismo de mamíferos, bem como novas formas de terapia contra estas intoxicações. A interação entre o mercúrio e o selênio tem sido bastante estudada e os resultados são controversos. Entretanto, poucos estudos envolvendo o papel do $(PhSe)_2$ na toxicidade induzida pelo $HgCl_2$ são encontrados na literatura. Desta forma, este trabalho visa elucidar o efeito do $(PhSe)_2$ frente ao dano induzido pelo $HgCl_2$ em camundongos, seja por meio de intoxicações agudas ou subcrônicas.

Objetivos Específicos

- Verificar os efeitos do $(PhSe)_2$, bem como de outros possíveis agentes terapêuticos (NAC e DMPS), na toxicidade aguda induzida pelo $HgCl_2$ em camundongos.

- Ampliar prévios estudos do grupo, com o objetivo de elucidar a toxicidade da interação $HgCl_2$ + $(PhSe)_2$, administrados de forma aguda em camundongos.

- Verificar o papel do pré-tratamento com $(PhSe)_2$ na toxicidade sub-crônica induzida pelo $HgCl_2$ em camundongos.

- Verificar o papel do $(PhSe)_2$, em comparação com o DMPS, na tentativa de reversão dos danos causados pela administração sub-crônica de $HgCl_2$ em camundongos.

3. REVISÃO BIBLIOGRÁFICA

3.1. Toxicologia dos metais

3.1.1. Aspectos gerais

Sabe-se que diversos metais são nutrientes requeridos pelo organismo, sendo considerados metais essenciais. O ferro, o zinco, o magnésio, o manganês e o selênio são exemplos destes metais. Entretanto, os metais essenciais, dependendo de suas concentrações, podem apresentar efeitos tóxicos em determinados organismos (Kabata-Pendias e Pendias, 1993). Outros metais, considerados metais pesados, como o mercúrio, o alumínio, o chumbo e o cádmio, não são essenciais para os seres vivos, podendo causar efeitos tóxicos nos organismos expostos a estes elementos (Bruins et al., 2000). Estes metais são amplamente encontrados em nosso ambiente e os seres humanos são expostos a tais metais a partir de inúmeras fontes, incluindo o ar, a água, o solo e os alimentos (Miller, 1998).

Inúmeras definições são encontradas na literatura para o termo "metal pesado". Algumas destas teorias baseiam-se na densidade atômica, no peso ou número atômico ou ainda nas propriedades químicas destes elementos. Constantemente este termo é empregado a um grupo de elementos que está associado a problemas de poluição e toxicidade. Em uma das definições descritas por Alloway e Aires (1993) o termo refere-se ao grupo de metais e metalóides com densidade atômica maior que 6 g/cm^3.

Em 2002, Duffs publicou um artigo argumentando contra o uso do termo "metal pesado", relatando mais de 25 diferentes definições descritas na literatura para o termo. O autor concluiu que a terminologia não deve ser usada, sugerindo nova definição baseada na avaliação da toxicidade potencial dos elementos metálicos e de seus componentes. Apesar disso, neste estudo o termo "metal pesado" será utilizado, uma vez que ainda não existe um consenso sobre o assunto e uma terminologia adequada para ser empregada.

Os metais pesados talvez sejam os agentes tóxicos mais conhecidos do homem. Há 2000 a.C., quando abundantes quantidades de chumbo eram obtidas de minérios, provavelmente, tenha sido o início da utilização deste metal pelo homem. Arsênio e mercúrio foram citados por Teofrastos de Erebus (387-372 a.C.) e por Plínio, o Velho (23-79 d.C.). Mais tarde, em 1815, o cádmio foi descoberto em minérios contendo carbonato e zinco (Salgado, 1996).

O acúmulo de metais pesados no organismo humano representa um risco significativo para a saúde, levando a uma grande variedade de patologias, como a anemia, o câncer, a insuficiência renal crônica, a hipertensão, a gota, a infertilidade masculina, a gengivite, entre outros (Miller, 1998). Os mecanismos moleculares pelos quais os metais tóxicos causam seus efeitos ainda não estão totalmente esclarecidos. Porém, sabe-se que a elevada toxicidade dos metais pesados está associada, ao menos em parte, aos seus efeitos pró-oxidantes e, conseqüentemente, as suas capacidades de contribuírem para a geração de EROs, como o radical hidroxil ($OH^•$), o ânion superóxido ($O_2^{•-}$) e o peróxido de hidrogênio (H_2O_2). O aumento da produção de EROs pode resultar na extensiva diminuição das defesas antioxidantes, resultando em uma condição conhecida como estresse oxidativo (Quig, 1998). Além disso, a toxicidade decorrente da exposição a metais pesados pode ser devida ao deslocamento de metais essenciais de seus sítios de ligação ou devida a interações químicas com biomoléculas endógenas, como os grupos sulfidrilas (-SH). Os efeitos tóxicos geralmente resultam na alteração da estrutura de lipídios, proteínas e ácidos nucléicos (Goering et al., 2002) e ainda interferência com o processo de fosforilação oxidativa e balanço osmótico (Hughes e Poole, 1989).

A terapia com agentes quelantes é a forma mais efetiva de tratamento para intoxicações com metais pesados (Vamnes et al., 2003). Além disso, como a exposição aos metais pesados resulta em estresse oxidativo, a utilização de agentes com ação antioxidante é bastante estudada (Sharma et al., 2005). Além das terapias exógenas, através de agentes quelantes e/ou agentes antioxidantes, o organismo também possui mecanismos de defesa endógenos, como por exemplo, as metalotioneínas (MT).

As MT foram primeiramente isoladas em 1957, ligadas ao cádmio, em rim de cavalo (Margoshes et al., 1957). As MT são proteínas citosólicas de baixo peso

molecular, que se ligam a vários metais essenciais (zinco, cobre, etc.) e metais pesados (mercúrio, arsênio e cádmio) (Foulkes, 1982). O papel biológico das MT tem sido relacionado ao controle homeostático do metabolismo do zinco e do cobre e com o processo de detoxificação de cádmio e mercúrio (Cousin, 1983). O mecanismo de proteção das MT na toxicidade induzida por metais pesados deve-se ao fato de que estas proteínas possuem grupos -SH em sua estrutura e podem se complexar com os metais através destes grupos (Miles et al., 2000). De fato, Suzuki et al. (1998) relataram que as MT podem proteger contra a toxicidade induzida por metais pelo seqüestro destes através dos grupos -SH da proteína. Outra hipótese é que as MT podem proteger os tecidos contra o dano oxidativo (Patrick, 2003). Os resíduos de cisteína das MT são facilmente oxidados, agindo como "scavengers" de radicais de oxigênio (Palmiter, 1998). Além disso, as MT têm importante função no Sistema Nervoso Central (SNC), protegendo-o contra os danos induzidos por interleucinas (Giralt et al., 2002a), ácido caínico (Carrasco et al., 2000), injúria física (Giralt et al., 2002b), entre outros.

3.1.2. Mercúrio

3.1.2.1. Histórico

O mercúrio é um metal líquido à temperatura ambiente, conhecido desde os tempos da Grécia Antiga. Seu nome homenageia o deus romano Mercúrio, que era o mensageiro dos deuses. Essa homenagem é devida à fluidez do metal. O símbolo Hg vem do latim "hydrargyrum" que significa prata líquida (Azevedo, 2003).

O mercúrio já é conhecido e usado pela humanidade há pelo menos 3500 anos. Em 2700 a.C., a utilização do mercúrio no processo de amalgamação do ouro já era conhecida pelos fenícios. Também se sabe que este metal era usado como medicamento e afrodisíaco na China e na Índia por volta de 500 a.C. (Azevedo, 2003).

Na Idade Média, o mercúrio foi amplamente utilizado na produção de amálgamas (ligas de metal), especialmente de prata e de ouro, mas a principal aplicação foi na alquimia. O uso do mercúrio e seus compostos para fins terapêuticos

persistiu até o século XVI no tratamento de quase todas as doenças até então conhecidas. Em meados do século XVII, o mercúrio elementar foi o primeiro elemento químico a ter controle de sua utilização devido à incidência de sintomas apresentados por mineradores (Azevedo, 2003). No Brasil, houve mineração de mercúrio na região de Ouro Preto até fins do século XVII. Estas antigas lavras subterrâneas encontram-se hoje exauridas e abandonadas.

Nos dias atuais, o mercúrio tem ampla aplicação no setor industrial, como na metalúrgica, petroquímica e produção de lâmpadas. O mercúrio também é utilizado atualmente nas áreas de garimpo para extração de ouro, especialmente na região da Amazônia (Malm, 1991).

3.1.2.2. Propriedades do mercúrio

O mercúrio é um metal obtido principalmente a partir do minério cinábrio (sulfeto de mercúrio). Este metal ocorre naturalmente no meio ambiente e existe em um grande número de espécies químicas. As principais espécies de mercúrio são: mercúrio elementar (ou vapor de mercúrio ou mercúrio metálico), mercúrio orgânico e mercúrio inorgânico. Essas diferentes espécies apresentam solubilidade, reatividade e toxicidade diferentes e, conseqüentemente, comportam-se de diferentes modos no meio ambiente, provocando distintos impactos nos ecossistemas e na saúde. As diferentes espécies do metal também influenciam no transporte do mercúrio dentro dos compartimentos ambientais (UNEP, 2002). As principais espécies de mercúrio presentes em amostras ambientais e biológicas são apresentadas na Tabela 1.

Tabela 1: Principais espécies químicas de mercúrio em amostras ambientais e biológicas

Mercúrio elementar		Hg^0
Espécie orgânica	Metilmercúrio	CH_3Hg^+
	Etilmercúrio	$C_2H_5Hg^+$
	Fenilmercúrio	$C_6H_5Hg^+$
	Dimetilmercúrio	$(CH_3)_2Hg$
Espécie inorgânica	Íon mercúrico	Hg^{2+}
	Íon mercuroso	Hg^+
	Sulfeto de mercúrio	HgS

Fonte: Morita et al. (1998)

O mercúrio elementar possui estado de oxidação zero (Hg^0) e é um líquido de elevada tensão superficial, inodoro e de coloração prateada. Este metal é pertencente ao Grupo IIB da tabela periódica com número atômico 80, massa atômica 200,59 e densidade 13.6 g/ml. Os pontos de fusão e ebulição são -38°C e 357°C, respectivamente (Salgado et al., 1996). O Hg^0 é capaz de dissolver o ouro, a prata, o chumbo e os metais alcalinos, formando ligas relativamente consistentes (amálgamas) (UNEP, 2002; Azevedo, 2003; Olivares, 2003).

Quando se liga ao carbono, através de ligação covalente C-Hg, o mercúrio forma compostos orgânicos chamados organomercuriais que são encontrados principalmente no solo e na água. Os compostos organomercuriais possuem átomos de carbono ligados ao mercúrio, formando compostos do tipo RHgX e RHgR'. R e R' são radicais alquilas, como por exemplo metil, etil e propil; e o X corresponde a uma variedade de ânions, como cloreto, acetato, cianeto, iodeto e fosfato (Salgado, 1996). A ligação carbono-mercúrio é quimicamente estável, não sendo rompida em água e nem por ácidos ou bases fracas (UNEP, 2002; Azevedo, 2003). Estes compostos são, em sua maioria, representados pelo metilmercúrio, que é a espécie mais tóxica do mercúrio.

Compostos inorgânicos de mercúrio, também chamados de sais de mercúrio, são formados a partir da combinação dos íons Hg(I) e Hg(II) com elementos como cloro,

enxofre e oxigênio, e se apresentam, em sua maioria, em forma de pó ou cristal branco, com exceção do sulfeto de mercúrio (HgS), que é vermelho e fica preto quando exposto à luz. Os sais de mercúrio são suficientemente voláteis para existirem como gás, porém sua solubilidade e reatividade levam a uma deposição mais rápida que o elemento mercúrio. Suas propriedades físico-químicas estão relacionadas ao ânion ao qual o metal está ligado. O sulfato de mercúrio, por exemplo, possui elevada solubilidade em água, enquanto o cloreto de mercúrio é bastante solúvel em solventes orgânicos (UNEP, 2002; Azevedo, 2003).

3.1.2.3. Fontes de Exposição

O mercúrio na sua forma natural surge da degradação da crosta terrestre a partir de vulcões, solos, florestas, lagos e oceanos abertos (Mason et al., 1994). Patra e Sharma (2000) relataram que dois terços dos compostos de mercúrio no ambiente são originados de fontes naturais e um terço é resultado de atividades humanas.

A exposição ambiental ao mercúrio é bastante comum. A grande poluição com mercúrio no ambiente resultou, principalmente, no aumento da contaminação das espécies vegetais e animais ao longo das cadeias alimentares. De fato, Magos e Clarkson (2006) demonstraram que a exposição de seres humanos ao mercúrio pode ocorrer através de alimentos. Dependendo do nível de contaminação, o ar e a água também podem se tornar importantes fontes de exposição ao metal. Além disso, o uso de combustíveis de origem fóssil pode aumentar os níveis de mercúrio no ar (Vimy e Lorscheider, 1985).

A elevada aplicação do mercúrio na indústria pode acarretar em exposição ocupacional a este metal. A ação tóxica do mercúrio na exposição ocupacional é conhecida desde a antiguidade, quando era usado para a decoração, em pinturas de castelos e no trabalho de mineração, que foi intensificado durante o Império Romano (Battigelli, 1983). A quantidade de mercúrio na atmosfera aumentou desde o início da revolução industrial (USEPA, 2003). Há diferentes formas de intoxicação. A mais comum durante a atividade laboral é a intoxicação crônica, mas também podem ocorrer acidentes, que provocam intoxicação aguda. Além disso, o mercúrio, por ser inodoro e

incolor, torna-se especialmente perigoso quando aquecido na forma de vapor. Embora o uso industrial do mercúrio tenha sofrido reduções (ANVISA, 2001), devido a um controle mais efetivo, concentrações altas ainda estão presentes em produtos industriais (Boening, 2000).

O mercúrio elementar é um líquido à temperatura ambiente e possui uma expansão volumétrica uniforme em ampla faixa de temperatura. Além disso, tem alta tensão superficial e não adere a superfícies vítreas. Devido a estas características, a forma elementar deste metal é utilizada em instrumentos de medição, como termômetros e barômetros. O mercúrio elementar também é utilizado para a produção de cloro, soda cáustica, equipamentos elétricos e eletrônicos (baterias, retificadores, relés, interruptores), tinta látex, amálgamas, lâmpadas de mercúrio, entre outros (Salgado, 1996; Broussard et al., 2002). No Brasil, o maior problema da contaminação do meio ambiente pelo mercúrio elementar é nas áreas de garimpos de ouro, principalmente na região Amazônica (Malm, 1991). Durante a queima do amálgama nos garimpos de ouro, a exposição aos vapores de mercúrio é bastante comum. Além das amálgamas presentes nos garimpos de ouro, outra forma de exposição ao mercúrio é através das amálgamas dentárias (UNEP, 2002).

Os compostos orgânicos de mercúrio são utilizados na agricultura (fungicidas, herbicidas, inseticidas), manufatura de papel, conservação de madeira, catalisador, entre outros (Andrade Filho et al., 2001). No entanto, a principal forma de intoxicação por metilmercúrio é a ingestão de peixes e mariscos que acumulam o metilmercúrio (UNEP, 2002; Azevedo, 2003). De fato, o metilmercúrio é a principal causa das doenças e intoxicações por mercúrio que causaram grandes desastres como o da Baía de Minamata no Japão, onde uma fábrica lançou metilmercúrio como resíduo nas águas da baía, contaminando os peixes e, conseqüentemente, a população. Um agravante para o problema da poluição é que o mercúrio inorgânico pode ser convertido a metilmercúrio e a dimetilmercúrio pela ação de microorganismos (bactérias metanogênicas), processo conhecido como biotransformação (Daughney et al., 2002). Este processo de metilação pode ocorrer tanto no solo quanto nos ambientes aquáticos (Azevedo, 2003).

A forma inorgânica do metal é utilizada em certos tipos de baterias, na produção de lâmpadas fluorescentes, manufatura de tintas, laboratório químico/fotográfico e produção de explosivos (Clarkson, 1997). Outras áreas de uso industrial do mercúrio inorgânico incluem a fabricação de plásticos, fungicidas e germicidas (Klaassen, 1996). Como dito anteriormente, ao atingir os ambientes aquáticos, o mercúrio inorgânico pode ser convertido, pela ação de microorganismos, em formas orgânicas do metal, as quais são extremamente tóxicas (Daughney et al., 2002).

3.1.2.4. Absorção, distribuição e excreção

A toxicocinética do mercúrio é dependente da espécie do metal ao qual o organismo está exposto. Assim, os processos de transporte serão influenciados pelo estado de oxidação e pela forma dos compostos de mercúrio (WHO, 1989; 1990; 1991).

A exposição ao mercúrio elementar ocorre principalmente quando respirado como vapor ou partículas de mercúrio, sendo absorvido pelos pulmões (UNEP, 2002). Sob condições normais de ventilação pulmonar, a absorção dos vapores de mercúrio pelos alvéolos pulmonares corresponde à cerca de 80% da sua concentração total no ambiente (Larini at al., 1997). A absorção cutânea do mercúrio elementar é muito pequena, mas pode causar dermatite nos casos de sensibilidade ao mercúrio (Azevedo, 2003). Já a ingestão de mercúrio elementar não produz efeitos adversos porque a absorção é muito pequena no trato gastrintestinal (inferior a 0,01 % da dose ingerida) (HSDB, 2000). O metilmercúrio é quase totalmente absorvido pelo trato gastrintestinal (90-100%) além de ser eliminado pelo leite materno, sendo, por conseqüência, uma via de exposição para o lactente (Greenwood et al., 1978). O mercúrio orgânico também é bastante absorvido pelas vias inalatória (cerca de 80 % dos vapores inalados) (WHO, 1991) e cutânea (WHO, 1989). Os compostos inorgânicos de mercúrio podem ser absorvidos através dos pulmões, com absorção dependente do diâmetro da partícula do aerossol inalado (WHO, 1991), e, com menor importância, através da pele (HSDB, 2000). Cerca de 2 a 10% do mercúrio inorgânico pode ser absorvido através do trato gastrintestinal (HSDB, 2000). Os sais de mercúrio (II) são usualmente mais tóxicos que

os de mercúrio (I), porque sua solubilidade em água é maior; portanto, eles são mais prontamente absorvidos a partir do trato gastrintestinal (Brasil, 1999).

O mercúrio elementar, após ser absorvido, é parcialmente oxidado a mercúrio iônico nos eritrócitos e nos tecidos. As formas orgânicas, lipossolúveis, concentram-se nos eritrócitos (Salgado et al., 1996). Já o mercúrio inorgânico distribui-se na corrente sangüínea, concentrando-se mais no plasma que nos eritrócitos. Os principais sítios de deposição de mercúrio no organismo são os rins e o cérebro para o mercúrio elementar, o cérebro para o mercúrio orgânico e os rins para o mercúrio inorgânico (Larini et al., 1997). O mercúrio elementar e o mercúrio orgânico, devido a serem lipossolúveis, atravessam rapidamente a barreira hematoencefálica e, por isso, atingem o tecido cerebral (Salgado, 1996). Além disso, as formas elementar e orgânica de mercúrio podem atravessar a barreira placentária (Salgado, 1996). Taxas menores de mercúrio ainda podem ser acumuladas no fígado, pulmão, coração, baço e intestino (Larini et al., 1997).

São possíveis quatro reações de biotransformação dos compostos de mercúrio: oxidação do mercúrio metálico a mercúrio divalente, envolvendo o sistema enzimático catalase-hidrogênio peroxidase (HSDB, 2000); redução de mercúrio divalente a mercúrio metálico (WHO, 1991); metilação do mercúrio inorgânico, através de microorganismos; e conversão do metilmercúrio a mercúrio divalente, devido ao rompimento da ligação carbono-mercúrio da molécula (Vahter et al., 1994). A transformação das formas elementar e orgânica na forma divalente do metal explica a presença de mercúrio inorgânico em fetos e no tecido cerebral de pessoas expostas às formas lipossolúveis deste metal (WHO, 1991; HSDB, 2000).

A excreção dos compostos de mercúrio se dá principalmente pelas vias urinária (formas elementar e inorgânica) e fecal (forma orgânica) (Larini et al., 1997). Após a exposição aos vapores de mercúrio, o metal é eliminado pela via renal na forma de Hg^{2+}, sendo a filtração glomerular prejudicada em razão da formação de complexos Hg-proteínas. O mercúrio elementar pode ainda ser excretado pelo ar exalado (HSDB, 2000). O metilmercúrio possui lenta eliminação. No cérebro e rins, esta eliminação leva um tempo considerável (até mesmo alguns anos) (Faro, 2000). A conversão do metilmercúrio para mercúrio inorgânico, permite explicar o processo de eliminação do

primeiro, que ocorre tanto em animais de experimentação quanto no homem, no qual se observa que 90% do composto ingerido é eliminado pelas fezes na forma inorgânica, já que esta forma de mercúrio é pouco absorvida através da membrana intestinal (Bjorkman et al., 1997). Neste processo, o metilmercúrio e o Hg^{2+}, por meio de secreção biliar, são complexados com a glutationa (GSH), além de outros peptídeos sulfidrílicos. O mercúrio pode ainda ser excretado através da saliva, lágrimas, suor e leite (Larini et al., 1997).

3.1.2.5. Toxicidade do mercúrio

A toxicidade induzida por compostos de mercúrio depende da natureza da exposição, da intensidade e da forma química deste metal (Diner, 2003).

O metilmercúrio é a forma mais tóxica de mercúrio, sendo responsável pelos danos mais importantes à saúde observados em humanos. Isto se deve, provavelmente, à sua lenta eliminação (Faro, 2000). O sistema nervoso central é o alvo principal do metilmercúrio, o qual afeta, principalmente, áreas específicas do cérebro, como cerebelo e lobos temporais. A intoxicação por metilmercúrio se caracteriza por comprometimento de nervos periféricos (parestesia de extremidades, lábios e língua, exacerbação dos reflexos tendíneos profundos e neurite periférica) e comprometimento do SNC (constrição do campo visual, alteração auditiva, distúrbios da fala e confusão mental), principalmente a nível cerebelar (ataxia, disartria e distúrbio da marcha). Insuficiência renal também tem sido observada após exposições ao metilmercúrio (WHO, 1990; Lebel et al.,1998).

O sistema respiratório é principalmente afetado pela exposição ao vapor de mercúrio elementar, em níveis elevados e em curto prazo (ATSDR, 1989). Exposições ao vapor de mercúrio elementar acima de 1-2 mg/m^3, por algumas horas, pode causar bronquite aguda e pneumonia. Duas horas após a exposição aos vapores de mercúrio, observa-se dano no pulmão através da formação de uma membrana hialina e, finalmente, a ocorrência de fibrose pulmonar (Sigeyuki et al., 2000). Além disso, exposições crônicas ao mercúrio elementar podem causar danos ao tecido renal, atingindo o glomérulo e os túbulos renais (ATSDR, 1992).

Sabe-se que o mercúrio inorgânico pode causar vários danos ao organismo, tais como danos ao sistema reprodutivo (Underwood, 1977; Anderson et al., 1992), ao sistema hepático (Huang et al., 1996), ao sistema pulmonar (Sener et al., 2007) e ao sistema renal (Perottoni et al., 2004), sendo este último o alvo primário da forma inorgânica do metal (Emanuelli et al., 1996; Clarkson, 1997). Além disso, o cérebro também pode ser afetado pelo mercúrio inorgânico, já que esta espécie de mercúrio pode ser formada no cérebro como um metabólito de outras formas de mercúrio (Vahter et al., 1994). É importante ressaltar que, no presente estudo, os efeitos do mercúrio inorgânico (cloreto de mercúrio) sobre os sistemas hepático, renal, hematológico e imunológico serão enfatizados.

O fígado é um órgão de grande importância tendo em vista suas diferentes funções: metabolismo, excreção, secreção, armazenamento, proteção, circulação e coagulação sanguínea. Dentre as funções citadas, uma das mais relevantes consiste na função de desintoxicação, através do sistema microssomal de biotransformação de xenobióticos (sistema citocromo P-450 ou CYP) (Motta et al., 2002). Estudos têm demonstrado que exposições a diferentes metais pesados causam danos ao tecido hepático (Borges et al., 2008; Mishra et al., 2008). Estes danos podem ser induzidos, em parte, pela geração de estresse oxidativo. De fato, Sener et al. (2007) demonstraram que a exposição ao cloreto de mercúrio causa a geração de EROs e redução de GSH no tecido hepático de ratos.

O rim é um órgão vital que desempenha papéis essenciais no organismo. As principais funções deste órgão são: regular a osmolaridade e volume de líquido corporal; eliminar substâncias tóxicas endógenas ou exógenas; manter o equilíbrio de eletrólitos e regular o equilíbrio ácido-básico. A unidade funcional do rim é o néfron, o qual é constituído por glomérulo, cápsula de Bowman, túbulo proximal, alça de Henle, túbulo distal e túbulo coletor (Sherwood, 2001). Devido à alta taxa de fluxo sanguíneo, o rim é um dos principais alvos da toxicidade induzida por xenobióticos (Hart and Kinter, 2005). Neste contexto, Yoshioka e Ichikawa (1989) demonstraram que a geração de EROs pelo mercúrio pode comprometer a função renal, diminuindo a taxa de filtração glomerular.

O rim é de grande importância quando se trata de exposições ao mercúrio, especialmente na sua forma inorgânica, uma vez que a excreção desta forma de mercúrio se dá principalmente via renal (Graeme e Pollock, 1998). Existem evidências que indicam que a captação do mercúrio pelo tecido renal envolve tanto a membrana basolateral (voltada para o sangue) quanto a membrana luminal (voltada para o lûmem tubular), principalmente nos túbulos proximais (Zalups, 2000). O principal mecanismo de captação dos íons mercúrio através da membrana basolateral é através de um sistema de transporte de ânions orgânicos. Já para a captação através da membrana luminal, parece que o transporte é feito através de transportadores de aminoácidos (Zalups, 2000). Baseado no fato de os íons mercúricos possuírem grande afinidade por grupos -SH (Zalups and Lash, 1994), sugere-se que uma significante fração dos íons mercúricos no plasma (após a exposição ao mercúrio inorgânico) esteja ligado a proteínas tiólicas de baixo peso molecular, antes de serem captados pela membrana basolateral. De fato, existem evidências que indicam que a maioria do mercúrio presente no plasma está ligada à albumina sérica, uma proteína sulfidrílica (Lau and Sarkar, 1979). Segundo alguns estudos, pode haver uma troca da albumina por GSH ou cisteína (Cys), ou outras moléculas tiólicas, e transporte dos complexos Hg-GSH ou Hg-Cys para dentro das células renais (Zalups e Barfuss, 1995a; b).

Os danos nas células sangüíneas ocasionados pela exposição ao mercúrio parecem estar relacionados com os efeitos hemolíticos induzidos por este metal. A indução de hemólise parece ser devido aos efeitos pró-oxidantes do mercúrio (Zolla et al., 1997; Brandão et al., 2005). Ribarov et al. (1982) demonstrou que os íons mercúricos podem interagir com componentes citoplasmáticos eritrocitários, ocasionando hemólise. O processo hemolítico parece ser uma conseqüência da inibição de enzimas citoplasmáticas antioxidantes, tais como a superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GPx) ou redução nas defesas antioxidantes não enzimáticas, como a GSH (Ribarov e Benov, 1981). Além disso, o complexo Hg^{+2}-hemoglobina pode causar a produção de EROs (Zolla et al., 1997). O processo de agregação plaquetária também pode ser alterado pela exposição ao mercúrio. Kumar (2000) demonstrou que o mercúrio e o arsênio aumentam a agregação plaquetária, e isto parece ocorrer através da inibição da adenilato ciclase ou

ativação da fosfodiesterase, com uma conseqüente redução nos níveis de 3'-5' adenosina monofosfato cíclico (AMPc) (Figura 1), o qual é um segundo mensageiro responsável, entre outras coisas, por inibir a agregação ou adesão plaquetária (Feinstein et al., 1985).
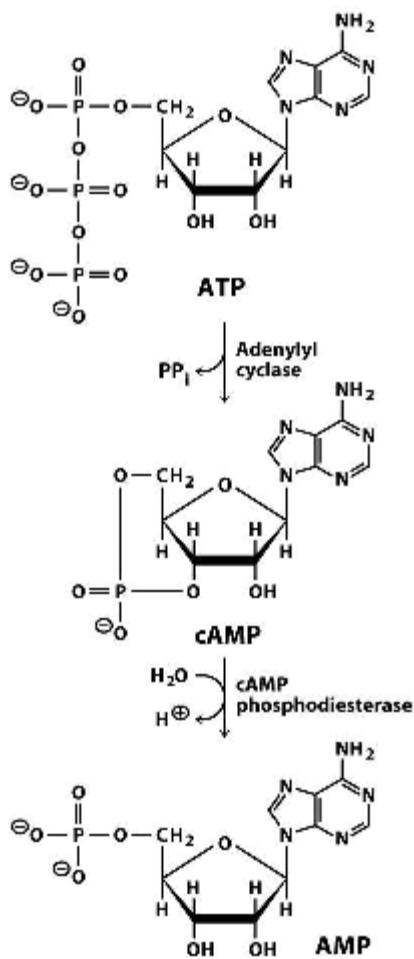
Figura 1: Formação e degradação do AMPc

A presença de alterações no sistema imunológico induzidas pela exposição ao mercúrio inorgânico é bastante relatada na literatura. Soleo et al. (1997) indicam que a exposição crônica a baixas concentrações de mercúrio inorgânico resulta em disfunções imunológicas. Segundo Zelikoff & Thomas (1998), a exposição a baixas concentrações de mercúrio, em suas várias formas químicas, pode induzir doenças auto-imunes e deprimir ou estimular o sistema imune em várias espécies animais. Num

primeiro momento, o mercúrio induz a auto-imunidade, ou seja, o sistema imunológico produz anticorpos contra suas próprias proteínas. Por meio deste mecanismo, o mercúrio lesa os rins pela produção de anticorpos que interferem com as proteínas das membranas renais. Estes anticorpos depositam-se nos rins causando, eventualmente, disfunção renal e o aparecimento de sintomas clínicos. Este é o mecanismo de indução de glomerulonefrite, observada em trabalhadores expostos ao mercúrio. O aumento nos níveis de imunoglobulinas, após exposição ao mercúrio, é um exemplo desta auto-imunidade (Silberg & Devine, 2000). Além disto, estudos recentes apontam a apoptose de linfócitos e monócitos induzida por mercúrio, como tendo um papel importante na disfunção imune causada por este metal (Ben-ozer et al., 2000; Shenker et al., 2000). De fato, linfócitos e monócitos tratados com mercúrio (tanto orgânico como inorgânico) exibem sinais funcionais e morfológicos de morte celular, como por exemplo, um profundo decréscimo na produção de adenina, alteração na síntese de fosfolipídeos, e um aumento de íons cálcio. Como estas células apresentam mudanças nucleares e de membrana que são consideradas características de uma célula em processo de morte celular programada, pode-se concluir que células linfóides tratadas com mercúrio são levadas à apoptose (Guo et al., 1998).

3.1.2.6. Mercúrio e estresse oxidativo

Como já descrito, o estresse oxidativo está bastante relacionado com a toxicidade induzida por metais pesados. Sob condições fisiológicas normais, as células produzem EROs por meio da redução do oxigênio molecular. A produção dos derivados tóxicos de oxigênio é aumentada como resultado de vários tipos de estresse (Foyer et al., 1994). A geração de EROs, tais como o radical hidroxil (OH$^\bullet$), o ânion superóxido (O$_2^{\bullet-}$), o peróxido de hidrogênio (H$_2$O$_2$) e o oxigênio singlete (^{1}O$_2$) tem demonstrado ser um dos agentes causadores de injúria nos tecidos. A toxicidade do mercúrio está associada com o estresse oxidativo, uma vez que este metal causa a geração de EROs e altera o sistema de defesa antioxidante celular (Hussain et al., 1999). De fato, o estresse oxidativo é bastante observado em intoxicações pelo mercúrio (Huang et al., 1996; Hoffman e Heinz, 1998; El-Demerdash, 2001). Segundo Lund et al. (1991), o

mercúrio estimula a lipoperoxidação por aumentar a formação de H_2O_2 na mitocôndria. Além disso, o mercúrio, similarmente a outros metais, pode participar das reações de Haber-Weiss e de Fenton e assim propiciar a formação de radicais $OH^{\bullet}$ (Halliwell e Gutteridge, 1990) (Figura 2), que iniciam o processo de peroxidação lipídica e de oxidação protéica. Esta geração de EROs também pode ser responsável por danos ao DNA e pela depleção dos grupos -SH de moléculas endógenas (Lund et al., 1991; 1993).

Seqüência da reação de Fenton

$$O_2^- + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^- + OH^{\bullet}$$
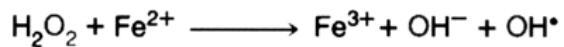
Reação de Haber-Weiss

$$H_2O_2 + O_2^- \longrightarrow O_2 + OH^- + OH^{\bullet}$$
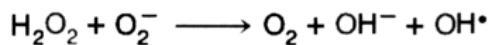
Figura 2: Seqüência das reações de Fenton e Haber-Weiss

Além de gerar diretamente EROs, como descrito acima, o mercúrio também pode causar um aumento indireto nas EROs. Tem sido demonstrado que o mercúrio inibe a atividade de diversas enzimas antioxidantes (enzimas "scavengers" de radicais livres), como a catalase (CAT), superóxido dismutase (SOD) e glutationa peroxidase (GPx) (Benov et al., 1990) (Figura 3), que podem proteger contra danos celulares, injúria tecidual, câncer e numerosas patologias relacionadas com a idade (Hussain et al., 1999). Outra enzima que pode ser alterada pela exposição ao mercúrio é a glutationa S-transferase (GST). Esta é uma enzima de metabolização de fase II (Hayes et al., 2005), que catalisa a conjugação de xenobióticos com a GSH, formando um conjugado menos tóxico e mais facilmente excretado. Dessa forma, a exposição à xenobióticos, como o mercúrio, pode causar uma ativação nesta enzima (Brambila et al., 2002). Além disso, a GST é relatada por apresentar ação antioxidante (Casalino et al., 2004).

A exposição ao mercúrio pode, também, reduzir as defesas antioxidantes não-enzimáticas como, por exemplo, a GSH (Zalups et al., 2000). A redução nos níveis de GSH ocorre porque o mercúrio possui grande afinidade por grupos –SH de biomoléculas endógenas (Clarkson, 1997). Deste modo, ele pode se complexar com estruturas que contêm estes grupos -SH, como a Cys e a GSH (Zalups, 2000). Resultados controversos têm sido obtidos com relação aos efeitos do mercúrio nos níveis de GSH. Zalups (2000) demonstrou que baixas doses de mercúrio podem aumentar os níveis de GSH renal, enquanto que altas doses deste metal podem reduzir os níveis de GSH renal. O aumento na concentração de GSH observado após a exposição aos compostos de mercúrio pode estar relacionado com o aumento na atividade da enzima glutationa redutase (GR) (Figura 3), o qual é relatado pela literatura (Lash e Zalups, 1996). Outra defesa antioxidante não-enzimática alterada pela exposição ao mercúrio é a vitamina C (Lund et al., 1993; Perottoni et al., 2004).
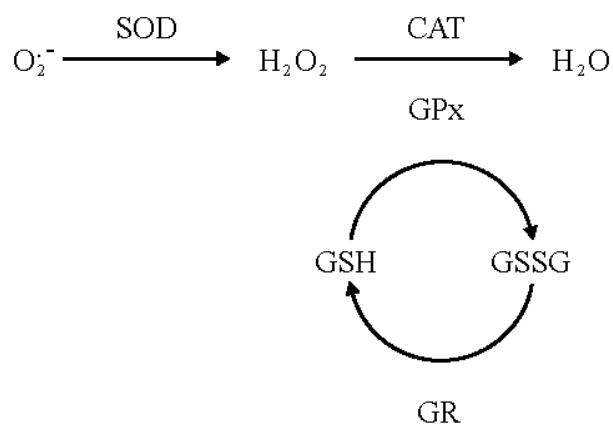
Figura 3: Funções das enzimas antioxidantes

Devido a sua natureza sulfidrílica, a enzima δ-aminolevulinato desidratase (δ-ALA-D) pode ser inibida por uma variedade de substâncias que possuam a propriedade química de oxidar grupos –SH, como por exemplo o mercúrio (Emanuelli et al., 1996). A enzima δ-ALA-D faz parte da rota biossintética do grupo heme (Figura 4). O heme (ferroprotoporfirina) faz parte da estrutura de proteínas que participam do transporte e armazenamento de oxigênio (hemoglobina e mioglobina, respectivamente); do

transporte de elétrons (citocromos a, b e c); da biotransformação de xenobióticos (citocromo P450) e do sistema de proteção contra peróxidos (catalases e peroxidases) (Timbrell, 1991). A inibição da δ-ALA-D, portanto, pode prejudicar a rota biossintética do heme, resultando em conseqüências patológicas (Sassa et al., 1989; Goering, 1993). Além da redução na síntese do heme, a inibição desta enzima pode resultar no acúmulo do substrato ácido δ-aminolevulínico (ALA) no sangue, com conseqüente aumento na excreção urinária do mesmo. O acúmulo de ALA pode estar relacionado com a superprodução de espécies reativas de oxigênio (Bechara et al., 1993). Além disso, o ALA gerado no fígado e na medula óssea pode atravessar a barreira hemato-encefálica, apresentando efeitos neurotóxicos (Becker et al., 1971; Cutler et al., 1979). Além da enzima δ-ALA-D, o mercúrio pode inibir a atividade de outras enzimas sulfidrílicas, como por exemplo a Na^+-K^+-ATPase, a qual também é sensível a agentes oxidantes (Anner et al., 1990). Esta é uma enzima de membrana responsável pelo transporte ativo dos íons sódio e potássio (Doucet, 1988).
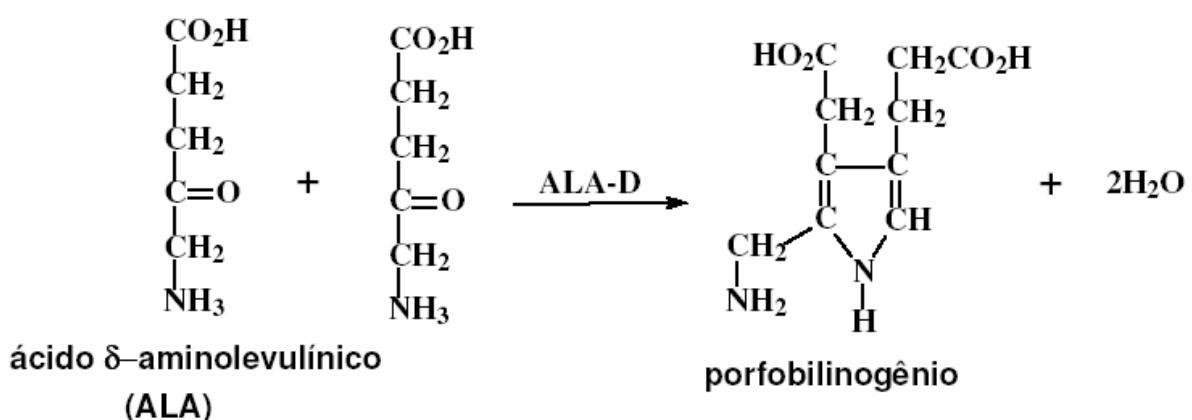
Figura 4: Condensação assimétrica de 2 moléculas do ALA,

catalisada pela enzima δ-ALA-D

3.2. Agentes quelantes

3.2.1. Aspectos gerais

A intoxicação por elementos metálicos requer um tipo de tratamento específico, sendo a terapia por meio de agentes quelantes a forma mais efetiva (Flora e Kumar, 1993). A quelação refere-se à coordenação de dois ou mais átomos de um ligante ao átomo metálico central, formando uma molécula denominada de quelato. Os agentes quelantes devem apresentar algumas características: devem ser capazes de penetrar nos tecidos e alcançar os metais através de afinidade química; devem formar compostos atóxicos; e devem formar compostos hidrossolúveis, facilitanto a excreção do quelato pela urina (Benite et al., 2002). Os compostos sulfidrílicos como o dimercaprol (British Anti-Lewisite, BAL), a D-penicilamina (Klaassen, 1996) e derivados do dimercaprol como o ácido *meso*-2,3-dimercaptosuccínico (DMSA) (Endo e Sakata, 1995; Flora, 1999; Frumkim et al., 2001) e o ácido 2,3-dimercapto-1-propanosulfônico (DMPS) (Pingree et al., 2001) são os agentes quelantes mais utilizados na terapêutica contra as intoxicações metálicas. Estes compostos, além de aumentarem a excreção do metal tóxico, também reduzem a toxicidade dos metais por impedir a ligação destes em moléculas celulares alvo (Aposhian et al., 1995).

Os primeiros relatos do uso de agentes quelantes em casos de intoxicações datam da época da Segunda Guerra Mundial, na Inglaterra. Stocken e Thompson, em 1946, descreveram o uso do 2,3-dimercaptopropanol (BAL) como um antídoto para intoxicações pelo dicloro-vinil arsênio. Este composto é um potente agente tóxico presente em gases de guerra, conhecido como Lewisite, o qual é capaz de atuar nos pulmões, nos rins, outros órgãos internos ou outras superfícies do corpo. Segundo relatos, o BAL proporcionava 100% de sobrevivência em animais expostos topicamente ao Lewisite quando comparado a outros quelantes menos efetivos, como o monotiol 2-mercaptoetanol (Stocken e Thompson, 1946). Entretanto, devido à sua lipossolubilidade, o BAL pode atravessar a membrana celular e atingir os espaços intracelulares (Andersen, 1989), causando redistribuição de metais, como o arsênio, o metil mercúrio (Hoover e Aposhian, 1983), o mercúrio inorgânico (Aaseth et al., 1995) e o chumbo (Cory-Slechta et al., 1987) dos órgãos periféricos para o cérebro. Uma vez

que a utilização do BAL apresenta diversas limitações devido à sua toxicidade, outros agentes quelantes, potencialmente menos tóxicos, têm sido investigados (Keith et al., 1997). Derivados estruturais do BAL, o DMPS e o DMSA, são mais hidrossolúveis e possivelmente menos tóxicos do que o BAL (Andersen, 1989).

É importante ressaltar que a utilização de agentes quelantes pode apresentar efeitos adversos. O tratamento prolongado com agentes quelantes pode causar distúrbios hematopoiéticos (Flora e Kumar, 1993), desequilíbrio do metabolismo celular e das sínteses de DNA, ácido ribonucléico (RNA) e proteínas (Fischer et al., 1975), ou ainda alteração da homeostase dos elementos traços (Cantilena e Klaassen, 1982). Existem, entretanto, alguns efeitos adversos decorrentes da utilização de agentes quelantes. Como o mecanismo terapêutico dos agentes quelantes envolve a ligação e excreção dos metais tóxicos do organismo, estes compostos podem interagir com metais endógenos essenciais, em especial o zinco, podendo ocasionar uma redistribuição ou até mesmo uma elevação na excreção dos mesmos (Cantilena e Klaassen, 1981). De fato, dados da literatura mostraram que o DMSA e o DMPS aumentam a excreção urinária de cobre e zinco em ratos (Khandelwal et al., 1987) e em humanos (Smith et al., 2000). Como descrito anteriormente, os metais endógenos são componentes essenciais de muitos sistemas enzimáticos, como a enzima δ-aminolevulinato desidratase (δ-ALA-D), que requer íons zinco para sua atividade catalítica máxima (Jaffe et al., 2004).

3.2.2. Ácido 2,3-dimercapto-1-propanosulfônico (DMPS)

O DMPS (Figura 5) tem sido usado na antiga URSS desde 1958 e encontra-se disponível, comercialmente na Europa, como DIMAVAL®. Além disso, este composto tem sido utilizado na Alemanha para o tratamento de intoxicações por mercúrio (Campbell et al., 1986). Este quelante apresenta-se como sólido cristalino e estável (Aposhian et al., 1992) e é facilmente administrado por via oral (Aposhian et al., 1996). A DL_{50} em camundongos tratados com DMPS é de 5,22 mmol.kg^{-1} (Aposhian et al., 1981). Já camundongos tratados com BAL apresentam DL_{50} de 0,73 mmol.kg^{-1}. Devido

a estas características, o DMPS é considerado menos tóxico que o BAL (Aposhian et al., 1992). De fato, o DMPS foi descrito como uma droga efetiva no tratamento de intoxicações por mercúrio (Kostygou, 1958), substituindo o BAL (Toet et al., 1994; Campbell et al., 1986; Cherian et al., 1988), apresentando menor toxicidade local e sistêmica (Hruby e Donner, 1987) e não causando redistribuição de mercúrio para o cérebro de ratos (Buchet e Lauwerys, 1989; Aposhian et al., 1996).

O DMPS apresenta dois grupos -SH vicinais e caracteriza-se pela maior solubilidade em água (Nadig et al., 1985) e limitada solubilidade lipídica (Aposhian et al., 1983). A presença de dois grupos -SH vicinais é reconhecida como a estrutura essencial para a eficácia do agente quelante (Muckter et al., 1997). Em intoxicações agudas por mercúrio, por exemplo, uma das terapêuticas fundamentais é a utilização de compostos que apresentem na sua estrutura grupos -SH (Schwartz et al., 1992; Klaassen, 1996). Estes compostos ditiólicos possuem a capacidade de complexar este metal pesado e aumentar a velocidade de excreção renal e biliar (Jugo, 1980; Shimada et al., 1993).

Como mencionado anteriormente, Zalups (2000) demonstrou que, após a exposição ao mercúrio inorgânico, os íons mercúricos são acumulados principalmente nas células dos túbulos proximais. O DMPS parece retirar o mercúrio do compartimento intracelular e levar para o lúmem tubular, aumentando a excreção deste metal na urina devido a falta de captação luminal dos conjugados de mercúrio (Zalups et al., 1998). Entretanto, Sweetman (2002) demonstrou que o DMPS possui a capacidade de quelar elementos essenciais como o cobre, o cromo e o zinco. Além disto, a associação entre mercúrio e DMPS parece apresentar efeitos pró-oxidantes. Nogueira et al. (2003b) demonstraram que a inibição na atividade da enzima δ-ALA-D hepática de camundongos, induzida por mercúrio e cádmio, foi potencializada tanto pelo DMPS quanto pelo DMSA. Neste contexto, estudos têm demonstrado que complexos formados entre o mercúrio e moléculas tiólicas podem apresentar atividades pró-oxidantes maiores do que os componentes isolados (Miller e Woods, 1993; Putzer et al., 1995).
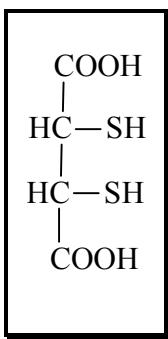
Figura 5: Estrutura do ácido 2,3-dimercapto-1-propanosulfônico (DMPS)

3.3. Agentes Antioxidantes

3.3.1. Aspectos gerais

Conforme descrito anteriormente, a intoxicação por metais pesados pode causar estresse oxidativo, seja através da redução de defesas antioxidantes ou pela geração de EROs (Hussain et al., 1999; Wang et al., 2004). Uma vez que os antioxidantes possuem uma importante função protegendo as células contra o estresse oxidativo, a utilização destes compostos pode ser eficaz em proteger contra as intoxicações pelo mercúrio. Neste contexto, muitos compostos com potencial antioxidante têm sido testados na terapia de intoxicações em que o estresse oxidativo esteja envolvido (Sener et al., 2003; Meotti et al., 2004; Santos et al., 2004; 2005).

3.3.2. N-acetilcisteína (NAC)

A NAC (Figura 6) é considerada um importante agente terapêutico e é comumente utilizada na prática clínica (Repine et al., 1997), sendo empregada como agente mucolítico, administrado por inalação, e também como tratamento de intoxicação por acetoaminofeno (Ziment, 1986; Borgström et al., 1986). Além disso, tem

sido demonstrado que a NAC possui efeito antimutagênico e anticarcinogênico em animais experimentais (De Flora et al., 1986a; b).

Sabe-se que a NAC pode estimular a síntese de GSH (Moldeus et al., 1986), uma vez que contém grupos tióis em sua estrutura. A NAC é uma doadora destes grupos tióis, atuando como precursora da Cys intracelular, indispensável para a síntese de GSH, assegurando-lhe sua função normal na proteção celular. Desta forma, a NAC é utilizada como antioxidante em condições de estresse oxidativo (Moldeus et al., 1986; Aruoma et al., 1989). Vários estudos têm indicado que a NAC também possui atividade quelante com relação a diversos metais pesados (Banner et al., 1986).

A utilização da NAC como agente antioxidante ou quelante, entretanto, requer certos cuidados, já que Ritter et al. (2004) descreveram que o uso da NAC pode ter algumas limitações e apresentar efeitos pró-oxidantes, devido à facilidade com que interage com o ferro. Além disso, alguns estudos têm demonstrado que a co-administração de moléculas tiólicas de baixo peso molecular (Cys, homocisteína e NAC) com o mercúrio aumenta a concentração do metal no tecido renal, sugerindo que o complexo tiol-mercúrio seria mais facilmente transportado pelos túbulos renais do que o metal somente (Zalups & Barfuss, 1998).
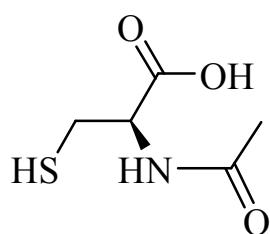
Figura 6: Estrutura da N-acetilcisteína (NAC)

3.3.3. Selênio

O selênio (Se) foi descoberto em 1817, pelo químico sueco Jöns Jacob Berzelius. Esse elemento é um calcogênio do grupo 16 da tabela periódica, podendo apresentar-se sob quatro estados de oxidação: selenato (Se^{+6}), selenito (Se^{+4}), selênio elementar (Se^0) e seleneto (Se^{-2}). O selênio compartilha propriedades químicas e

físicas com o enxofre. Esta similaridade permite que o selênio substitua o enxofre, promovendo interações selênio-enxofre nos sistemas biológicos. Por outro lado, as diferenças nas propriedades fisico-químicas entre selênio e enxofre constituem a base de seus papéis biológicos específicos (Stadtman, 1980).

O selênio é um elemento traço essencial, cuja essencialidade nutricional foi demonstrada em 1957, em ratos (Schwartz e Foltz, 1957). Nos últimos anos, têm sido descrito 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-Alarcón e López-Martinez, 2000). Neste contexto, a suplementação de dietas com selênio, tanto para animais quanto para humanos, tem sido aceita pela comunidade científica. Para humanos, a Junta de Alimentação e Nutrição da Academia de Ciências dos Estados Unidos propõe uma ingestão diária de 50-200 μg, a qual é considerada segura e saudável para adultos. Este elemento pode ser encontrado nos seguintes alimentos: castanha-do-pará, alho, cebola, brócolis, cogumelos, cereais, pescados, ovos e carnes (Dumont et al., 2006). Por outro lado, sabe-se que a concentração alimentar requerida de selênio é muito próxima da dose que pode ser tóxica (Oldfield, 1987). De fato, estudos demonstraram que altas doses de selênio podem ser citotóxicas, uma vez que possuem a habilidade de oxidar grupos –SH e gerar radicais livres (Barbosa et al. 1998; Nogueira et al. 2004).

Este calcogênio apresenta um grande número de funções biológicas, sendo a mais importante como antioxidante. Sabe-se que moléculas contendo selênio, como por exemplo o $(PhSe)_2$, podem ser melhores nucleófilos (e portanto antioxidantes) do que os antioxidantes clássicos (Arteel e Sies, 2001). As pesquisas recentes têm procurado estabelecer a função e a biologia molecular de selenoproteínas. Já é conhecido que o selênio está presente como resíduo de selenocisteína no sítio ativo das enzimas glutationa peroxidase (Wingler e Brigelius-Flohé, 1999), tioredoxina redutase (Holmgren, 1985), 5'-deiodinase (Behne e Kyriakopoulos, 1990) e selenoproteína P (Ursini et al., 1990). A atividade redox do selênio tem fundamental importância para o sítio catalítico dessas enzimas.

Muitos trabalhos têm reportado uma interação entre o selênio e o mercúrio em mamíferos, podendo o calcogênio ter um papel protetor contra a toxicidade induzida

pelo mercúrio (Sasakura e Suzuki, 1998; El-Demerdash, 2001; Farina et al., 2003; Moretto et al., 2005a; b). De fato, evidências demonstram que o selênio pode se associar ao mercúrio e com a selenoproteína P e formar um complexo ternário inerte reduzindo, desta forma, a toxicidade do metal (Yoneda e Suzuki, 1997). Entretanto, observamos, em um estudo *in vitro*, um efeito tóxico da interação entre selênio (como selenito de sódio) e mercúrio (como cloreto de mercúrio) em eritrócitos humanos (Brandão et al., 2005). Outros trabalhos também relatam a presença de efeitos tóxicos decorrentes da interação entre selênio e mercúrio (Magos, 1991; Farina et al., 2004).

3.3.3.1. Absorção, distribuição e excreção

Nos mamíferos, o selênio parece ser rapidamente absorvido no duodeno, seguido pelo jejuno e íleo. Além do trato gastrointestinal, o selênio pode ser absorvido por tecidos cutâneos e inalação. Estas duas últimas vias de absorção estão relacionadas com a exposição e intoxicação ocupacional por compostos de selênio (Whanger et al., 1976).

Após a absorção, os maiores níveis de selênio estão localizados nos eritrócitos, baço, coração, unha e esmalte de dentes (Martin e Gerlack, 1972). Na intoxicação crônica em animais, o selênio é depositado principalmente nos rins e fígado, seguido pelo pâncreas, baço e pulmões (Wilber, 1980). A primeira evidência de metabolização dos compostos de selênio em animais foi determinada após um longo período de tratamento com o selenito de sódio. Os animais apresentavam odor gárlico característico, que posteriormente demonstrou-se ter sido causado pelo seleneto de dimetila (Klayman e Gunther, 1973). Este metabólito pode ser resultado do processo de detoxificação do selênio, o qual envolve uma série de metilações dependentes da S-adenosilmetionina (Hoffman e McConnell, 1986).

O selênio pode ser excretado por três vias: urina, fezes e ar expirado. A excreção urinária deste composto pode auxiliar em casos de intoxicações ou de exposições a altos níveis deste elemento (Valentine et al., 1978). Recentemente, foi demonstrado que dentro dos níveis normais de selênio, ou seja, não tóxicos, a principal forma encontrada na urina é como seleno-açúcar. Entretanto, nos casos de doses

tóxicas de selênio, o marcador biológico encontrado na urina é o trimetilselenônio (Suzuki et al., 2006). Em indivíduos expostos acidentalmente a altos níveis de selênio, pode ser realizada a detecção do composto volátil seleneto de dimetila (Mozier et al., 1988).

3.3.3.2. Disseleneto de difenila $(PhSe)_2$

A partir da década de 30, os organocalcogênios têm sido alvos de interesse para os químicos orgânicos em virtude da descoberta de aplicações sintéticas (Petragnani et al., 1976; Comasseto, 1983), sendo importantes intermediários e reagentes muito utilizados em síntese orgânica (Paulmier, 1986; Braga et al., 1996; 1997). Conseqüentemente, o risco de contaminação ocupacional por organocalcogênios tem motivado estudos toxicológicos. Outro aspecto relevante é a tentativa crescente de desenvolvimento de compostos organocalcogênios que possuam atividades biológicas e aplicações farmacológicas (Parnham e Graf, 1991; Nogueira et al., 2003a).

Durante as últimas décadas, o interesse nos compostos orgânicos de selênio tem sido intensificado, principalmente devido ao fato de que uma variedade destes compostos possui propriedades farmacológicas (Nogueira et al., 2004). Em especial, destaca-se o disseleneto de difenila $(PhSe)_2$ (Figura 7), um composto orgânico de selênio bastante lipofílico e que apresenta inúmeras propriedades farmacológicas (Nogueira et al., 2004). De fato, estudos em animais de laboratório têm demonstrado que este composto apresenta propriedades antiúlcera (Savegnago et al., 2006), antiinflamatória e antinociceptiva (Savegnago et al., 2007), antidepressiva e ansiolítica (Savegnago et al., 2008), neuroprotetora (Ghisleni et al., 2003), hepato-protetora (Borges et al., 2005a; 2006), anti-hiperglicêmica (Barbosa et al., 2006) e pode retardar o desenvolvimento de câncer (Barbosa et al., 2008). Além disso, o $(PhSe)_2$ apresenta efeitos antioxidantes em diversos modelos de toxicidade induzida por estresse oxidativo (Meotti et al. 2004; Luchese et al., 2007), incluindo exposições ao cádmio (Santos et al., 2004; 2005; Borges et al., 2008). Nogueira et al. (2004) sugerem que, pelo fato do disseleneto possuir atividades semelhantes as da glutationa peroxidase, este composto é um bom candidato a ser um agente antioxidante. Adicionalmente, também foi

demonstrado que o (PhSe)$_2$ pode apresentar atividade complexante em animais expostos ao cádmio (Santos et al., 2005).

Santos et al. (2006), entretanto, relatam que quando administrado de forma sub-cronica, em associação com o cádmio, o (PhSe)$_2$ pode potencializar o dano tecidual induzido pelo metal. Além disso, outros trabalhos têm demonstrado efeitos tóxicos do (PhSe)$_2$. Inibições nas atividades das enzimas δ-ALA-D (Nogueira et al., 2003c) e Na$^+$, K$^+$-ATPase (Borges et al., 2005b) são observadas e o potencial pró-oxidante do (PhSe)$_2$ parece estar envolvido nestes efeitos. Efeitos neurotóxicos do (PhSe)$_2$ também são relatados (Nogueira et al., 2003d), incluindo a indução de convulsões (Prigol et al., 2008). O (PhSe)$_2$ pode ainda afetar o sistema glutamatérgico em plaquetas humanas (Borges et al., 2004) e de ratos (Nogueira et al., 2001) e dados da literatura também indicam que o (PhSe)$_2$ pode causar toxicidade em fetos de ratas tratadas com este composto (Favero et al., 2005; Weis et al., 2007).

Embora existam muitos estudos na literatura avaliando o efeito da interação entre selênio e mercúrio em mamíferos, estudos utilizando o disseleneto de difenila são escassos. Moretto et al. (2005a) demonstraram, em um estudo *in vitro*, que o disseleneto de difenila pode proteger contra as alterações na fosforilação de proteínas induzidas pelo metilmercúrio em cérebro de ratos jovens. Já em um outro estudo *in vitro*, Moretto et al. (2005b) demonstraram que o disseleneto de difenila não foi eficaz em proteger contra a inibição da captação de glutamato induzida pelo metilmercúrio e cloreto de mercúrio no córtex cerebral de ratos jovens. Além disso, verificamos, em um estudo *ex vivo*, que o (PhSe)$_2$, embora tenha apresentado poucos efeitos terapêuticos, foi efetivo em reduzir os níveis de uréia aumentados pelo cloreto de mercúrio em camundongos (Brandão et al., 2006), sugerindo um efeito benéfico deste organocalcogênio no tecido renal.
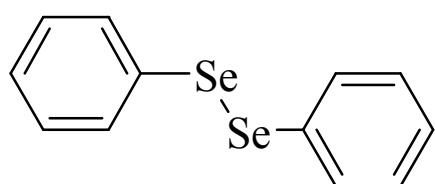
Figura 7: Estrutura do (PhSe)$_2$

4. ARTIGOS CIENTÍFICOS E MANUSCRITOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos científicos e manuscritos, os quais se encontram assim organizados. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas encontram-se nos próprios artigos e manuscritos. Os artigos estão dispostos da mesma forma que foram publicados nas revistas científicas (artigos 1 e 2). O manuscrito 2 está disposto da mesma forma que foi submetido para avaliação. O manuscrito 1 encontra-se em fase de redação.

4.1. DMPS e N-acetilcisteína induzem toxicidade renal em camundongos expostos ao mercúrio

4.1.1. Artigo 1

DMPS AND N-ACETYLCISTEYNE INDUCED RENAL TOXICITY IN MICE EXPOSED TO MERCURY

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DMPS and *N*-acetylcysteine induced renal toxicity in mice exposed to mercury

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Abstract

Acute effects of mercuric chloride ($HgCl_2$) were evaluated on mice. Mice received a single dose of $HgCl_2$
(4.6 mg/kg, subcutaneously) for three consecutive days. Thirty minutes after the last injection with $HgCl_2$,
mice received one single injection of 2,3-dimercapto-1-propanesulfonic acid (DMPS) or *N*-acetylcysteine
(NAC) or diphenyl diselenide $(PhSe)_2$. DMPS, NAC and $(PhSe)_2$ were utilized as therapy against mercury
exposure. At 24 h after the last $HgCl_2$ injection, blood, liver and kidney samples were collected. δ-Ami-
nolevulinate dehydratase (δ-ALA-D) and Na^+, K^+ ATPase activities, thiobarbituric acid-reactive sub-
stances (TBARS), non-protein thiols (NPSH) and ascorbic acid concentrations were evaluated. Plasma
aspartate (AST) and alanine (ALT) aminotransferase activities, as well as urea and creatinine levels were
determined. The group of mice exposed to Hg + $(PhSe)_2$ presented 100% of lethality. Exposure with
$HgCl_2$ caused a decrease on the body weight gain and treatments did not modify this parameter. δ-ALA-D,
AST and ALT activities, TBARS, ascorbic acid levels and NPSH (hepatic and erythrocytic) levels were not
changed after $HgCl_2$ exposure. $HgCl_2$ caused an increase in renal NPSH content and therapies did not
modify these levels. Mice treated with $(PhSe)_2$, Hg + NAC and Hg + DMPS presented a reduction in
plasma NPSH levels. Creatinine and urea levels were increased in mice exposed to Hg + NAC, while Hg
+ DMPS group presented an increase only in urea level. Na^+, K^+ ATPase activity was inhibited in mice
exposed to Hg + DMPS and Hg + NAC. In conclusion, therapies with $(PhSe)_2$, DMPS and NAC fol-
lowing mercury exposure must be better studied because the formation of more toxic complexes with
mercury, which can mainly damage renal tissue.

Introduction

Inorganic mercury is widely used in certain types
of batteries and continues to be an essential com-
ponent of fluorescent light bulbs (Clarkson 1997).
The toxic effects of mercury on human and animal
systems are well documented (WHO 1976, 1990).
It is known that mercuric chloride (Hg^+) can
stimulate lipid peroxidation by enhancing H_2O_2
formation in mitochondria (Lund *et al*. 1991).

A method for the detoxification of mercury that
is widely recommended is its transformation into a
chelate complex (Jones 1994). 2,3-Dimercapto-1-
propanesulfonic acid (DMPS), a chelating agent,
has been shown to be effective for the treatment of
mercury intoxication in animals and humans
(Aposhian *et al*. 1995). However, Nogueira and
colaborators (2003c) have reported that DMPS
inhibited hepatic δ-aminolevulinate dehydratase
(δ-ALA-D) activity from mice and the inhibition

390

potency of DMPS is increased in the presence of mercury and cadmium. Since mercury induces oxidative stress (Huang *et al.* 1996), it is believed that antioxidants should be one of the most important components of an effective treatment against mercury intoxication.

N-acetylcysteine (NAC), a sulfhydryl containing antioxidant (Moldeus *et al.* 1986), is possibly one of the most widely investigated compounds that has beneficial effects on clinical conditions in which free radicals are involved (Berend 1985). Selenium is an essential element with physiological antioxidant properties, appearing as a selenocysteine (Bock *et al.* 1991), a structural component of several enzymes involved in peroxide decomposition, including glutathione peroxidase (Flohé *et al.* 1973; Rotruck *et al.* 1973) and phospholipid hydroperoxide glutathione peroxidase (Ursini *et al.* 1982). Organic forms of selenium have been suggested as possible antioxidant agents because they exhibit glutathione peroxidase-like activity (Nogueira *et al.* 2004). Conversely, Nogueira *et al.* (2003a) demonstrated that diphenyl diselenide (PhSe)$_2$ inhibits δ-ALA-D activity from human blood by interacting with SH groups of the enzyme.

Because Hg$^+$ has great affinity for SH groups of endogenous biomolecules, which may contribute to its toxicity (Clarkson 1997), δ-ALA-D and Na$^+$, K$^+$-ATPase, sulfhydryl containing enzymes, could be sensitive to mercury exposure. Accordingly, Rocha *et al.* (1993, 1995) reported that δ-ALA-D activity was inhibited after mercuric chloride administration. This enzyme plays a fundamental role in most aerobic organisms by participating in heme biosynthesis pathway (Sassa 1998) and its inhibition can lead to δ-ALA accumulation, which in turn can enhance the generation of free radicals, aggravating oxidative damage to cellular components (Pereira *et al.* 1992; Bechara 1996).

In addition, Na$^+$, K$^+$-ATPase could also be sensitive to oxidizing agents (Thévenod & Friedmann, 1999; Folmer *et al.* 2004; Borges *et al.* 2005). Na$^+$, K$^+$-ATPase is an enzyme embedded in the cell membrane and responsible for the active transport of sodium and potassium ions. This process regulates the cellular Na$^+$/K$^+$ concentrations and hence their gradients across the plasma membrane, which are required for vital functions such as membrane co-transports, cell volume

regulation and membrane excitability (Doucet 1988; Jorgensen 1986). Several reports have shown the effects of metals such as mercury (Klonne *et al.* 1988; Anner *et al.* 1992) cadmium and lead (Pedrenho *et al.* 1996; Garcia & Corredor 2004) on Na$^+$, K$^+$-ATPase activity.

In the present study, we examined the effects of DMPS, (PhSe)$_2$ and NAC on acute mercury-poisoning in mice. Thereby, we evaluated the effect of mercury and therapies on δ-ALA-D and Na$^+$, K$^+$-ATPase activities, thiobarbituric acid-reactive substances (TBARS), non-protein thiol and ascorbic acid levels on mice tissues. The parameters that indicate hepatic (aspartate (AST) and alanine (ALT) aminotransferase activities in plasma) or renal (urea and creatinine levels) damage were also examined.

Materials and methods

Chemicals

Mercuric chloride (HgCl$_2$) was obtained from Merck (Darmstadt, Germany). 2,3-Dimercapto-1-propanesulfonic acid (DMPS), *N*-acetylcysteine (NAC), δ-aminolevulinic acid (δ-ALA) and *p*-dimethylaminobenzaldehyde were purchased from Sigma (St. Louis, MO, USA). Diphenyl diselenide was synthesized according to Paulmier (1986). Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of diphenyl diselenide (99.9%) was determined by GC/HPLC. All other chemicals were of analytical grade and obtained from standard commercial suppliers. (PhSe)$_2$ was dissolved in DMSO (dimethylsulfoxide).

Animals

Male adult Swiss albino mice (30–35 g) from our own breeding colony were used. The animals were kept on separate animal rooms, on a 12 h light/dark cycle, at a room temperature of 22 °C, 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, Federal University of Santa Maria, Brazil.

Exposure

A group of six to eight mice was usually tested in each experiment. Mice received one daily injection of mercuric chloride ($HgCl_2$), subcutaneously, at the dose of 4.6 mg/kg (dissolved in saline at 0.46 mg/ml) for three consecutive days (Emanuelli *et al.* 1996; Perottoni *et al.* 2004b). Thirty minutes after the last injection with mercury, mice received one injection of DMPS at the dose of 400 μmol/kg intraperitonealy (i.p) (Santos *et al.*, 2004) or NAC (300 mg/Kg, i.p) or diphenyl diselenide (100 μmol/kg, s.c) (Nogueira *et al.* 2003b).

The protocol of mice treatment is given below:

Group 1 (control): saline (s.c.) + DMSO (s.c.) + saline (i.p.)

Group 2 (DMPS): saline (s.c.) + DMPS (i.p.) + saline (i.p.)

Group 3 (PhSe)$_2$: saline (s.c.) + (PhSe)$_2$ (s.c.) + saline (i.p.)

Group 4 (NAC): saline (s.c.) + NAC (i.p.) + DMSO (s.c.)

Group 5 (Hg): $HgCl_2$ (4.6 mg/kg, s.c.) + DMSO (s.c.) + saline (i.p.)

Group 6 (Hg + DMPS): $HgCl_2$ (4.6 mg/kg, s.c.) + DMPS (i.p.) + saline (i.p.)

Group 7 (Hg + (PhSe)$_2$): $HgCl_2$ (4.6 mg/kg, s.c.) + (PhSe)$_2$ (s.c.) + saline (i.p.)

Group 8 (Hg + NAC): $HgCl_2$ (4.6 mg/kg, s.c.) + NAC (i.p.) + DMSO (s.c.)

At 24 h after the last $HgCl_2$ injection, the blood samples were collected directly from the ventricle of the heart in animals anesthetized. Subsequently, mice were sacrificed and liver and kidney were removed.

Mortality index

The effects of DMPS, (PhSe)$_2$ and NAC on mortality index of mice exposed to mercury were evaluated 24 h after therapies.

Body weight

Effects of DMPS and NAC on body weight gain of mice exposed to mercury were evaluated. The body weight gain of mice was monitored for the whole course of the experiment.

δ-ALA-D activity

δ-ALA-D activity was assayed by the method of Sassa (1982) by measuring the rate of product (porphobilinogen) formation except that 45 mM potassium phosphate buffer, pH 6.4 and 2.5 mM of aminolevulinic acid (ALA) were used (Barbosa *et al.* 1998). Incubations were carried out for 30 (liver) and 60 min (kidney) at 39 °C. The reaction product was determined using modified Ehrlich's reagent at 555 nm, with a molar absorption coefficient of 6.1×10^4 per M for the Ehrlich-porphobilinogen salt.

Determination of non-protein thiols (NPSH)

NPSH in kidney, liver, erythrocytes and plasma were determined by the method of Ellman (1959). To determine NPSH in liver and kidney, the homogenate was centrifuged at 4000 × *g* at 4 °C for 10 min and the supernatant (500 μl) was mixed (1:1) with 10% trichloroacetic acid (500 μl). After centrifugation, the protein pellet was discarded and free – SH groups were determined in the clear supernatant.

The erythrocyte samples (300 μl) were hemolyzed with 10% Triton (100 μl) and precipitated with 200 μl of 10% trichloroacetic acid. After centrifugation, free – SH groups were determined in the supernatant. NPSH determination in plasma was carried out without sample treatment.

Determination of thiobarbituric acid-reactive substances (TBARS)

Lipid peroxidation was performed by the formation of TBARS during an acid-heating reaction as previously described by Draper and Hadley (1990). Briefly, the samples were mixed with 1 ml of 10% TCA and 1 ml of 0.67% thiobarbituric acid subsequently they were heated in a boiling water bath for 15 min. The absorbance was read at 532 nm and the data expressed as nmol malondialdehyde (MDA)/g tissue.

Ascorbic acid determination

Ascorbic acid determination was performed as described by Jacques-Silva *et al.* (2001). Protein (tissues) was precipitated in 10 volumes of a cold 4% trichloroacetic acid solution. An aliquot of

392

sample (300 μl), in a final volume of 1 ml of the solution, was incubated at 38 °C for 3 h, then 1 ml H_2SO_4 65% (v/v) was added to the medium. The reaction product was determined using a color reagent containing 4.5 mg/ml dinitrophenyl hydrazine and $CuSO_4$ (0.075 mg/ml).

Aspartate (AST) and alanine aminotransferase (ALT) activities

Plasma AST and ALT enzymes were used as the biochemical markers for the early acute hepatic damage. AST and ALT activities were determined using a commercial Kit (LABTEST, Diagnostica S.A, Minas Gerais, Brazil).

Urea and creatinine levels

Renal function was analyzed using a commercial Kit (LABTEST, Diagnostica S.A, Minas Gerais, Brazil) by determining plasma urea and creatinine.

Na^+, K^+-ATPase activity

Immediately after the mouse sacrifice, the kidney was removed and the homogenate was prepared in 0.05 M Tris–HCl buffer (pH 7.4). The homogenate was centrifuged and supernatant was used for assay of protein Na^+, K^+-ATPase. The reaction mixture for Na^+, K^+-ATPase activity assay contained 6 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl, 1 mM EDTA and 40 mM Tris–HCl, pH 7.4, in a final volume of 500 μl. The reaction was initiated by the addition of ATP to a final concentration of 3.0 mM. Controls were carried out under the same conditions with the addition of 1 mM ouabain. Na^+, K^+-ATPase activity was calculated by the difference between the two assays. Released inorganic phosphate (Pi) was measured by the method of Fiske & Subbarow (1925).

Statistical analysis

Data are expressed as mean ± S.E.M. Statistical analysis was performed to compare treatment groups to respective control groups using a one-way analysis of variance followed by the Duncan's multiple range test when appropriate. Values of $P < 0.05$ were considered statistically significant. For comparing the mortality index, a non-parametric chi-square test was applied.

Table 1. Effects of DMPS, (PhSe)$_2$ and NAC on mortality index of mice exposed to mercury.

	Mortality index
control	0/16
DMPS	0/16
(PhSe)$_2$	0/16
NAC	0/16
Hg	1/19
Hg + DMPS	2/19
Hg + (PhSe)$_2$	23/23[a, b]
Hg + NAC	2/20

[a]Denoted $P < 0.01$ as compared to the control group (chi-square test).
[b]Denoted $P < 0.01$ as compared to the Hg group (chi-square test).

Results

Mortality index

Results indicated that mice exposed to mercury plus (PhSe)$_2$ presented 100% of lethality (Table 1). For this reason, other parameters were not evaluated in this group (Hg + (PhSe)$_2$). In the other groups, there was no significant difference in the mortality index.

Body weight

The body weight gain of the mice was monitored for the whole course of the experiment. Mice exposed to mercury presented a reduction in the body weight gain when compared to the control group. Therapies with NAC and DMPS were not effective in restoring the body weight (Table 2).

δ-ALA-D activity

Results demonstrated that acute mercury exposure did not affect renal and hepatic δ-ALA-D activities. Therapies with DMPS and NAC did not modify enzyme activity when compared to the control and Hg groups (data not shown).

Lipid peroxidation

Renal and hepatic malondialdehyde levels, a parameter of TBARS formation, remained unchanged after mercury exposure. Therapies did not modify MDA levels when compared to the control and Hg groups (data not shown).

Table 2. Effects of DMPS and NAC on body weight gain of mice exposed to mercury.

	Body weight gain (g)
control	0.55 ± 0.58 (1.6)
DMPS	-0.48 ± 0.64 (−1.4)
(PhSe)$_2$	-0.81 ± 0.64 (−2.3)
NAC	-0.31 ± 0.49 (−0.9)
Hg	-1.85 ± 0.56 (−5.2)[a]
Hg + DMPS	-1.91 ± 0.97 (−5.6)[a]
Hg + NAC	-2.46 ± 0.98 (−6.9)[a]

Data are mean ± S.E.M. from six to eight animals in each group. ()% of the body weight change.
[a]Denoted $P < 0.05$ as compared to the control group (one-way ANOVA/Duncan).

Ascorbic acid concentration

Mercury exposure did not modify renal and hepatic ascorbic acid levels. DMPS and NAC therapies did not change ascorbic acid levels when compared to the control and Hg groups (data not shown).

Non-protein thiols (NPSH)

Results indicated that mercury exposure increases renal NPSH levels (186.61%) when compared to the control group ($P < 0.01$). Therapies did not change renal NPSH levels in comparison to the Hg group (Figure 1). Hepatic NPSH levels remained unchanged after mercury exposure and therapies

did not modify these levels (Figure 1). Mercury expo- sure and therapies did not change erythrocytes NPSH content (Figure 2). Mice exposed to (PhSe)$_2$ presented a decrease in plasma NPSH levels (30.19%) when compared to the control group ($P < 0.05$). Hg–DMPS and Hg–NAC groups also presented a decrease in plasma NPSH levels (about 30%) when compared to the control group ($P < 0.05$) (Figure 2).

Plasma AST and ALT activities

Neither mercury nor therapies with DMPS and NAC altered AST and ALT activities when compared to the control group (Table 3).

Urea and creatinine levels

It is surprising that the urea and creatinine concentrations are not significantly elevated in Hg treated mice. The absolute values (76.16 mg/dL for urea and 0.51 mg/dl for creatinine) are elevated though not significantly due to the large scatter (Table 3). Conversely, Hg + DMPS and Hg + NAC groups presented an increase in urea level (101.88 and 127.90%, respectively) when compared to the control group ($P < 0.01$). One-way analysis yielded an increase in urea level for Hg + DMPS and Hg + NAC groups (45.42 and 64.17%, respectively) when compared to the Hg
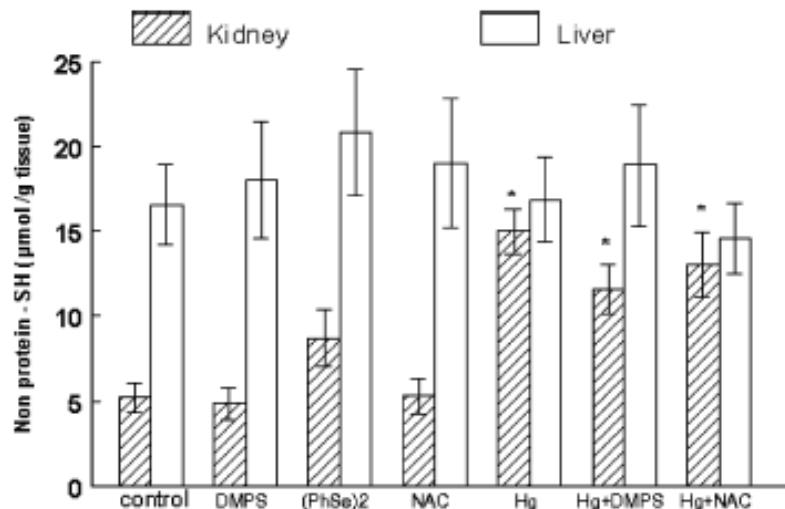
Figure 1. Effects of NAC and DMPS on non-protein thiol group content in liver and kidneys of mice exposed to mercury. Data are reported as mean ± S.E.M. of six to eight animals per group. (*) Denoted $P < 0.01$ as compared to the control group (one-way ANOVA/Duncan).

394
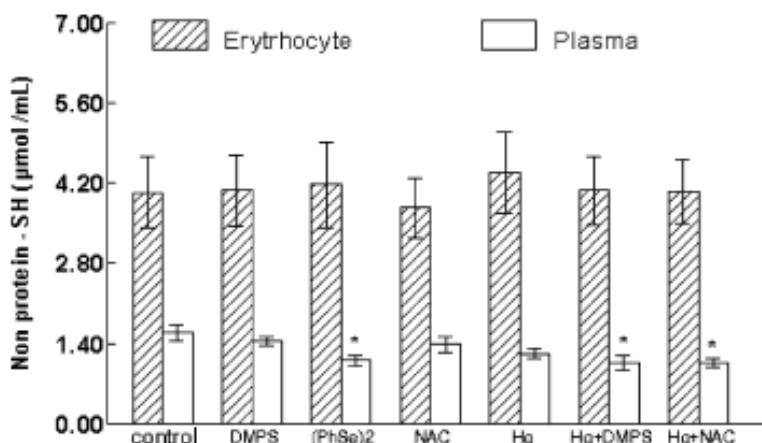
Figure 2. Effects of NAC and DMPS on non-protein thiol group content in erythrocytes and plasma of mice exposed to mercury. Data are reported as mean ± S.E.M. of six to eight animals per group. (*) Denoted $P < 0.05$ as compared to the control group (one-way ANOVA/Duncan).

Table 3. Effects of DMPS and NAC on urea and creatinine levels and on AST and ALT activities of mice exposed to mercury.

	Urea (mg/dl)	Creatinine (mg/dl)	AST (IU/l)	ALT (IU/l)
Control	54.86 ± 3.30	0.24 ± 0.023	114.33 ± 10.99	33.11 ± 3.08
DMPS	49.85 ± 5.38	0.33 ± 0.08	118.80 ± 16.99	33.20 ± 2.22
(PhSe)2	35.21 ± 2.97	0.32 ± 0.075	151.49 ± 40.04	28.40 ± 2.90
NAC	56.05 ± 5.21	0.40 ± 0.063	117.75 ± 28.75	36.25 ± 7.40
Hg	76.16 ± 10.89	0.51 ± 0.158	111.80 ± 19.39	23.55 ± 4.07
Hg + DMPS	110.75 ± 13.43[a, b]	0.62 ± 0.11	103.14 ± 19.24	28.85 ± 2.25
Hg + NAC	125.03 ± 14.68[a, b]	1.13 ± 0.23[a,b]	174.75 ± 18.30	37.77 ± 12.95

Data are mean ± S.E.M. from six to eight animals in each group.
[a]Denoted $P < 0.01$ as compared to the control group (one-way ANOVA/Duncan).
[b]Denoted $P < 0.05$ as compared to the mercury group (one-way ANOVA/Duncan).

group ($P < 0.05$) (Table 3). The results also demonstrated that mice exposed to Hg + NAC presented an increase in creatinine levels (370.83 and 121.57%, respectively) when compared to the control ($P < 0.01$) or Hg ($P < 0.05$) groups (Table 3).

Na^+, K^+-ATPase activity

Mercury exposure did not change renal Na^+, K^+-ATPase activity (Figure 3). Conversely, Na^+, K^+-ATPase activity was inhibited in mice exposed to Hg + DMPS and Hg + NAC (44.74 and 47.54%, respectively) in comparison to the control group ($P < 0.05$). One-way analysis revealed that Hg + DMPS and Hg + NAC groups presented Na^+, K^+-ATPase activity reduced

(45.29 and 48.07%, respectively) when compared to the Hg group ($P < 0.05$).

Discussion

Inorganic mercury has a non-uniform distribution after absorption, being accumulated mainly in kidneys (Emanuelli *et al.* 1996; Klaassen 1996). In the current study, mercury toxicity was evidenced by the reduction in the body weight gain after mercury exposure, whereas therapies with DMPS or NAC were not effective in restoring the body weight in mice exposed to mercury. In addition, we observed for the first time that mice exposed to mercury and treated with (PhSe)$_2$ presented 100% of lethality, suggesting a toxic synergistic effect
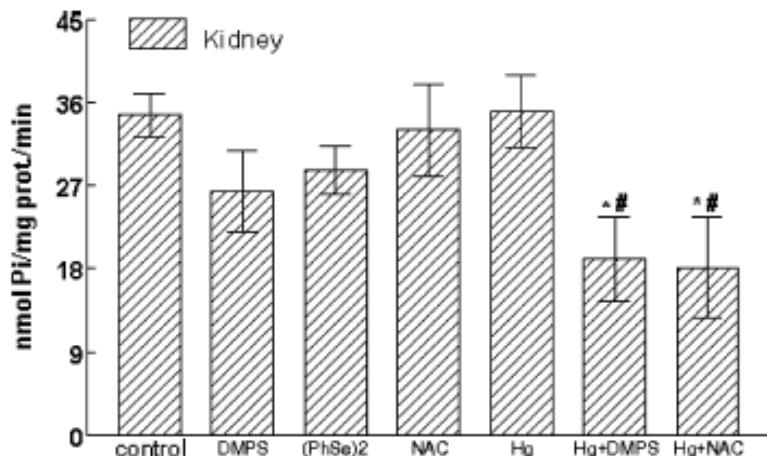
Figure 3. Effects of NAC and DMPS on renal Na$^+$, K$^+$-ATPase activity of mice exposed to mercury. Data are reported as mean ± S.E.M. of six to eight animals per group. (*) Denoted $P < 0.05$ as compared to the control group (one-way ANOVA/Duncan). (#) Denoted $P < 0.05$ as compared to the mercury group (one-way ANOVA/Duncan).

between these two compounds. Since mercury and (PhSe)$_2$ did not present renal and hepatic toxicity when given alone, we can infer that toxicity observed in mice simultaneously exposed to Hg and (PhSe)$_2$ is related to a complex formed between Hg and (PhSe)$_2$, which could have pro-oxidant activity. In addition, we have demonstrated that (PhSe)$_2$ presents chelating activity in mice exposed to cadmium (Santos *et al.* 2005b). Thus, the complex formed between cadmium and (PhSe)$_2$ appears to be different from Hg–(PhSe)$_2$ because diphenyl diselenide protected against toxicity induced by cadmium (Santos *et al.* 2005a), whereas Hg–(PhSe)$_2$ complex induced lethality in mice.

Our results also demonstrated that acute mercury exposure did not change δ-ALA-D activity and TBARS levels. These results are, at least in part, in accordance with previous studies that did not verify alterations in hepatic δ-ALA-D activity and TBARS levels in rats after mercury exposure (Perottoni *et al.* 2004a, b). Accordingly, Farina *et al.* (2003) have reported that acute mercuric chloride exposure did not alter renal and hepatic δ-ALA-D activity, as well as TBARS levels 24 after mercury poisoning.

In this study, renal NPSH levels were increased in mice after mercury exposure and were not modified by treatment with DMPS and NAC. Mercury is able to increase glutathione reductase (GR) activity under *in vivo* conditions (Lash & Zalups 1996). This increment can be related, at least in part, to the direct oxidative effects of

mercury on endogenous glutathione (GSH), which leads to the enhancement in the GR activity. Although renal GR activity was not measured in our protocols, the observed increase in renal NPSH levels can be interpreted as a pathophysiological response to preserve the homeostasis of intracellular thiol status. Another important data found in this study was that (PhSe)$_2$, Hg + DMPS and Hg + NAC groups presented decreased NPSH levels in plasma, but not in erythrocytes. Previous studies have demonstrated that GSH content varies in different tissues and even within different intracellular compartments, it can be inferred that characteristic redox states exist in different tissues. Thus, the variation between plasma and erythrocytes NPSH levels can be explained by differences in some of the factors that modulate the GSH content. For instance, GSH levels in cells are primarily dependent upon the rates of biosynthesis and utilization in oxidation/reduction reactions. Amounts of the precursor amino acids as cysteine and the activity of the enzyme glutamate–cysteine ligase are the key factors affecting GSH synthesis (Rebrin *et al.*, 2005).

Mercury exposition associated with therapies (DMPS and NAC) induced renal toxicity that was clearly evidenced by an increase in urea and creatinine levels. Regarding the renal parameters evaluated, NAC seems to be the most hazardous therapy utilized in this study. Moreover, the association of Hg with DMPS or NAC inhibited the renal Na$^+$, K$^+$-ATPase activity, supporting

396

the renal toxicity induced by both therapies. Na^+, K^+-ATPase is a sulfhydryl-containing enzyme, it is probable that the complex formed between Hg and DMPS or between Hg and NAC causes a Na^+, K^+-ATPase inhibition by oxidizing sulfhydryl groups critical for the enzyme activity. Accordingly, chemical interactions between metals (cadmium and mercury) and thiols have been described earlier (Rivera *et al.* 1989; Aposhian and Aposhian 1990). Nogueira and co-workers (2003c) have reported the formation of a complex (metal–chelator) between cadmium or mercury and DMPS. In addition, previous studies have reported that mercury–thiol complexes present pro-oxidant activity higher than isolated components (Miller & Woods 1993, Putzer *et al.* 1995).

N-acetylcysteine (NAC) is a thiol-containing antioxidant (Moldeus *et al.* 1986) and ROS scavenging (Aruoma *et al.* 1989). Some studies have indicated that NAC has also chelating activity with regard to diverse heavy metals (Banner *et al.* 1986). Thus, DMPS, NAC and $(PhSe)_2$ could exert their toxic effects due to the formation of complexes with mercury, which would be more toxic than the isolated components.

The hypothesis that Hg–DMPS or Hg–NAC complexes are transported by renal tubules more easily than Hg and therefore develop a stronger toxicity must be considered. In fact, several authors have reported that renal uptake of inorganic mercury involves a mechanism localized along the proximal tubule and appears to be dependent on the organic anion transport system (Zalups & Lash 1994; Zalups & Minor 1995; Zalups & Barfuss 1998). This transport system does not transport large proteins, while that small molecular conjugates of mercury are likely transported (Zalups 1998). Thus, the organic anion transport system may transport inorganic mercury conjugated with thiols (GSH, cysteine, NAC or DMPS) and increase mercury toxicity.

Therefore, the results obtained indicated that mercury exposition associated with therapies (DMPS and NAC) caused renal toxicity, which was evidenced by an increase in urea and creatinine levels and an inhibition in renal Na^+, K^+-ATPase activity. In addition, $(PhSe)_2$ therapy caused 100 % of lethality in mice exposed to mercury. In conclusion, we believe that therapies with $(PhSe)_2$, DMPS and NAC following mercury exposure must be better studied because the formation of more toxic complexes with mercury, which can mainly damage renal tissue.

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398

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4.2. Disseleneto de difenila potencializa a nefrotoxicidade induzida pelo cloreto de mercúrio em camundongos

4.2.1. Manuscrito 1

DIPHENYL DISELENIDE POTENTIATES NEPHROTOXICITY INDUCED BY MERCURIC CHLORIDE IN MICE

Manuscrito em fase de redação

Diphenyl Diselenide Potentiates Nephrotoxicity Induced by Mercuric Chloride in Mice

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Abstract

Following our longstanding interest on the mechanisms involved in selenium toxicity, the aim of this study was to gain a better understanding on the toxicity of mercuric chloride ($HgCl_2$) and diphenyl diselenide $(PhSe)_2$ interaction. Mice received one daily dose of $HgCl_2$ (4.6 mg/kg, subcutaneously) for three consecutive days. Thirty minutes after the last $HgCl_2$ injection, they received a single dose of $(PhSe)_2$ (100 µmol/kg, subcutaneously). Five hours after $(PhSe)_2$ administration, blood and urine samples were collected and mice were killed and had their kidney and liver dissected. Parameters in plasma (urea, protein and erythropoietin), whole blood (hematocrit and hemoglobin) and urine (glucose and protein) were investigated. Acivities of δ-aminolevulinate dehydratase (δ-ALA-D), catalase (CAT), glutathione S-transferase (GST) and Na^+, K^+- ATPase, as well as levels of thiobarbituric acid-reactive substances (TBARS), ascorbic acid and mercury were determined. Results demonstrated that $(PhSe)_2$ did not modify mercury concentrations in tissues of mice exposed to $HgCl_2$. $HgCl_2$ + $(PhSe)_2$ exposure caused a decrease in renal GST and Na^+, K^+- ATPase activities and increased renal ascorbic acid and TBARS concentrations when compared to the $HgCl_2$ group. $(PhSe)_2$ potentiated the increase in plasma urea and urine glucose and protein levels caused by $HgCl_2$. $HgCl_2$ + $(PhSe)_2$ exposure caused a reduction in plasma protein levels and an increase in hemoglobin and hematocrit contents when compared to the $HgCl_2$ group. There was a significant decrease in hepatic CAT activity and an increase in TBARS levels in mice exposed to $HgCl_2$ + $(PhSe)_2$ when compared to the $HgCl_2$ group. In conclusion, $(PhSe)_2$ potentiated damage caused by $HgCl_2$ affecting mainly the renal tissue in mice.

Key-Words: diphenyl diselenide, kidney, liver, mercury, selenium, toxicity.

1- Introduction

The industrial use of mercury and its general toxic effects on human and animal systems are well known (Rao, 1997). In fact, mercury promotes the formation of reactive oxygen species (ROS) such as hydrogen peroxides (Hussain et al., 1999). Accordingly, mercury exposure has been demonstrated to induce lipid peroxidation detected by an increased concentration of thiobarbituric acid-reactive substances (TBARS) in liver, kidney, brain, and other tissues (Huang et al., 1996). Moreover, mercury exposure can cause inhibition of sulfhydryl enzymes such as δ-aminolevulinate dehydratase (δ-ALA-D) (Emanuelli et al., 1996) and Na^+, K^+- ATPase (Anner and Moosmayer, 1992).

Selenium is a structural component of several enzymes with physiological antioxidant properties, including glutathione peroxidase and thioredoxin reductase (Rotruck et al., 1973; Xia et al., 2002). Diphenyl diselenide, $(PhSe)_2$, an organoselenium compound, has been reported in view of its pharmacological properties such as anti-inflammatory, antinociceptive, anti-ulcer, neuroprotective and antioxidant in different experimental models (Nogueira et al., 2004; Barbosa et al., 2006; Borges et al., 2006; Savegnago et al., 2006, 2007). Consistent with the property of "double face" of selenium compounds, toxicological properties of $(PhSe)_2$ have been reported (Nogueira et al., 2004). Our research group have demonstrated that $(PhSe)_2$ inhibits δ-ALA-D from human blood (Nogueira et al., 2003a) and cerebral Na^+, K^+- ATPase activity (Borges et al., 2005) by interacting with SH groups of these enzymes.

The interaction between mercury and selenium in the body of mammals has been known for more than three decades. Since Parizek and Ostadalova (1967) found that the toxicity of inorganic mercury was decreased by simultaneous injection of selenite,

many studies have been carried out to examine the role of selenium in the detoxification of mercury, which have led to many hypotheses about the mechanism of this interaction (Cuvin-Aralar and Furness, 1991). Regarding the interaction between organic selenium and mercury, data on neuroprotection (Farina et al. 2003a) and pro-oxidative effects have been reported (Farina et al., 2004).

Our research group has studied the interaction between selenium and mercuric chloride ($HgCl_2$) (Brandão et al., 2005; Brandão et al., 2006). Using the same protocol applied in the present study, we have demonstrated that $(PhSe)_2$ potentiates toxicity induced by $HgCl_2$ in mice (Brandão et al., 2006), since 100% of animals treated with $HgCl_2$ + $(PhSe)_2$ died 24 hours after exposure. Following our longstanding interest on the mechanisms involved in selenium toxicity, the aim of this study was to extend our previous studies (Brandao et al. 2006) to gain a better understanding on the toxicity of the $HgCl_2$ + $(PhSe)_2$ interaction. Therefore, endpoints of toxicity were analyzed in kidney, liver, blood and urine of mice exposed to $HgCl_2$ + $(PhSe)_2$.

2- Materials and methods

2.1- Chemicals

Mercuric chloride ($HgCl_2$) was obtained from Merck (Darmstadt, Germany). δ-Aminolevulinic acid (δ-ALA), adenosine tri phosphate (ATP), 1-chloro-2,4-dinitrobenzene (CDNB) and *p*-dimethyl aminobenzaldehyde were purchased from Sigma (St. Louis, MO, USA). $(PhSe)_2$ was prepared according to Paulmier (1986). Analysis of the ^1H NMR and ^{13}C NMR spectra showed analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of $(PhSe)_2$ (99.9%) was determined by

GC/HPLC. All other chemicals were of analytical grade and obtained from standard commercial suppliers. $(PhSe)_2$ was dissolved in dimethylsulfoxide (DMSO).

2.2- Animals

Male adult Swiss albino mice (30 - 35 g) from our own breeding colony were used. The animals were kept on separate animal rooms, on a 12 hour light/dark cycle, at temperature of $22 \pm 2°C$, with free access to food and water. This study was approved by the Ethics and Animal Welfare Committee of Universidade Federal de Santa Maria.

2.3- Exposure

A group of six mice was usually tested in each experiment. Mice received a single daily injection of $HgCl_2$ subcutaneously (s.c.) at the dose of 4.6 mg/kg (10 ml/kg, dissolved in saline solution) for three consecutive days. The dose of $HgCl_2$ used was selected based on the fact that previous study from our laboratory showed that it induced mild or moderate damage in rodents (Emanuelli et al., 1996; Perottoni et al., 2004). Thirty minutes after the last injection of $HgCl_2$, mice received a single s.c. injection of 100 µmol/kg $(PhSe)_2$ (2.5 ml/kg, dissolved in DMSO) (Nogueira et al., 2003b). The doses of $HgCl_2$ and $(PhSe)_2$, as well as the exposure protocol employed were the same used in the study that demonstrated 100% lethality after 24 hours of exposure to the combination of both compounds (Brandão et al., 2006).

The protocol for mouse treatment is given below:

Group 1: saline + DMSO

Group 2: saline + $(PhSe)_2$

Group 3: HgCl$_2$ + DMSO

Group 4: HgCl$_2$ + (PhSe)$_2$

Since it was shown that mice exposed to HgCl$_2$ + (PhSe)$_2$ (group 4) did not survived more than five hours after (PhSe)$_2$ administration (Brandão et al., 2006), the present experiment was stopped five hours after the last injection. After exposure, urine and blood samples were collected in anesthetized mice. Urine samples were collected by bladder punction, when possible. Subsequently, mice were decapitated and liver and kidney removed, rapidly homogenized (1/10, w/v) in 50 mM Tris–HCl, pH 7.4 and centrifuged (2400×g) for 15 min. The sample supernatant (S1) was separated and used for biochemical assays.

2.4- δ-ALA-D activity

Renal and hepatic δ-ALA-D activity was assayed using the method described by Sassa (1982), measuring the rate of product (porphobilinogen) formation, except that 100 mM potassium phosphate buffer, pH 6.8 and 2.4 mM of aminolevulinic acid (ALA) were used (Barbosa et al., 1998). Incubations of S1 were carried out at 37°C for 60 min. The reaction product was determined using the modified Ehrlich's reagent at 555 nm, with a molar absorption coefficient of 6.1 x 10^4 per M for the Ehrlich-porphobilinogen salt.

2.5- Renal Na$^+$, K$^+$- ATPase activity

The S1 was used for the Na$^+$, K$^+$- ATPase activity assay. The reaction mixture used contained 6 mM MgCl$_2$, 100 mM NaCl, 20 mM KCl, 1 mM EDTA and 40 mM Tris-

HCl (pH 7.4) in a final volume of 500 μl. The reaction was initiated by addition of ATP at a final concentration of 3.0 mM. Controls were carried out under the same conditions with the addition of 1 mM ouabain. Na^+, K^+- ATPase activity was calculated by the difference between the two assays. Released inorganic phosphate (Pi) was measured (650 nm) by the method of Fiske and Subbarow (1925).

2.6- Glutathione S-transferase (GST) activity

GST activity was assayed in S1 of liver and kidney through the conjugation of glutathione with CDNB at 340 nm, as described by Habig et al. (1974).

2.7- TBARS determination

An aliquot (200 μl) of liver or kidney S1 was incubated at 95°C for 2 h. TBARS were spectrophotometrically determined at 532 nm, as described by Ohkawa et al. (1979).

2.8- Catalase (CAT) activity

CAT activity was determined in kidney and liver S1 by the decomposition of H_2O_2 at 240 nm, according to Aebi (1984).

2.9- Ascorbic acid determination

Ascorbic acid determination in kidney and liver was performed as described by Jacques-Silva et al. (2001). S1 was precipitated in 10 volumes of a cold 4% trichloroacetic acid solution. An aliquot of sample (300 μl), in a final volume of 1 ml of

solution, was incubated at 37°C for 3 h, and 1 ml H_2SO_4 65 % (v/v) was added to the medium. The reaction product was determined at 520 nm using a color reagent containing 4.5 mg/ml dinitrophenyl hydrazine and $CuSO_4$ (0.075 mg/ml).

2.10- Plasma urea and protein levels

Plasma urea was analyzed using a commercial Kit (LABTEST, Diagnostica S.A, Minas Gerais, Brazil). Plasma protein was assayed by the method of Bradford (1976).

2.11- Urine glucose and protein levels

Test strips (Multistix 10 SG1 Bayer1, Munich, Germany, 2002) were used to semiquantitatively measure the amount of glucose and protein in fresh urine samples. Since urine was collected by bladder punction, samples were insufficient for other more specific dosages.

2.12- Hemoglobin, hematocrit and erythropoietin

Blood hemoglobin concentration was determined by the cyanomethemoglobin procedure (Wintrobe, 1965). Micro Wintrobe hematocrit tubes and hematocrit centrifuge were used to determine the packed cell volume (PCV, or hematocrit value). Blood was centrifuged at 4000×g for 5 min and the hematocrit value was obtained by reading the packed cells volume on the graduated hematocrit tubes. Erythropoietin levels were determined by chemiluminescence.

2.13- Mercury concentration

Tissues (kidney and liver) were completely digested with 65% nitric acid (Suprapur, Merk). Renal and hepatic mercury concentration was analyzed by cold vapour atomic absorption spectrometry (CVAAS; model A Analyst 800, Perkin Elmer) coupled to a generator of hydrides (model FIAS Mercury/Hydride, Chemifold). Standard from Fluka was used to build up the standard curve (1-50 micrograms/l).

2.14- Statistical analysis

Data are expressed as means ± S.D. All results were analyzed by two-way ANOVA, followed by Duncan's Multiple Range Test, when appropriate. Main effects are presented only when the higher second order interaction was non-significant. Differences between groups were considered to be significant when $p < 0.05$. For urine glucose data, a non-parametric Kruskal–Wallis test, followed by Mann–Whitney U test was used.

3- Results

3.1- δ-ALA-D activity

Two-way ANOVA of renal δ-ALA-D activity data demonstrated a main effect of $HgCl_2$ ($p<0.05$). δ-ALA-D activity was not altered in mice treated with $HgCl_2$ + $(PhSe)_2$ when compared to the $HgCl_2$ group (Figure 1A). Hepatic δ-ALA-D activity was not modified by the different treatments (Table 1).

3.2- Renal Na^+, K^+- ATPase activity

A significant interaction between $HgCl_2$ and $(PhSe)_2$ on Na^+, K^+- ATPase activity was observed ($F_{1,19}$=14,52, $p<0.001$). Animals treated with $HgCl_2$ + $(PhSe)_2$ presented a reduction (~31%) in the enzyme activity when compared to the $HgCl_2$ group (Figure 1B).

3.3- GST activity

Two-way ANOVA of renal GST activity data yielded a significant main effect of $HgCl_2$ ($p<0.01$) and $(PhSe)_2$ ($p<0.05$). Renal GST activity was inhibited (~40%) in mice exposed to $HgCl_2$ + $(PhSe)_2$ when compared to those only exposed to $HgCl_2$ (Figure 1C). Hepatic GST activity was not modified by exposure to both compounds (Table 1).

3.4- TBARS determination

Two-way ANOVA revealed a significant interaction between $HgCl_2$ and $(PhSe)_2$ in renal TBARS levels ($F_{1,24}$=14.92, $p<0.001$). Animals exposed to $HgCl_2$ + $(PhSe)_2$ showed an increase (~116%) in renal TBARS levels when compared to those exposed to $HgCl_2$ (Figure 2A).

A significant interaction between $HgCl_2$ and $(PhSe)_2$ in hepatic TBARS levels was observed ($F_{1,27}$=23.91, $p<0.001$). Animals exposed to $HgCl_2$ + $(PhSe)_2$ showed an increase (~79%) in hepatic TBARS levels when compared to those exposed to $HgCl_2$ (Table 1).

3.5- CAT activity

Two-way ANOVA of renal CAT activity data yielded a main effect of $HgCl_2$ ($p<0.001$). $(PhSe)_2$ exposure did not modify the inhibition induced by $HgCl_2$ (Figure 2B).

Mice exposed to HgCl$_2$ + (PhSe)$_2$ showed an inhibition (~23%) in the hepatic CAT activity when compared to those exposed to HgCl$_2$ (Table 2).

3.6- Ascorbic acid concentration

Two-way ANOVA of renal ascorbic acid data yielded a significant interaction between HgCl$_2$ and (PhSe)$_2$ ($F_{1,26}$=15.96, p<0.001). Mice exposed to HgCl$_2$ + (PhSe)$_2$ showed an increase (~30%) in renal ascorbic acid levels when compared to those exposed to HgCl$_2$ (Figure 2C). In contrast, hepatic ascorbic acid content was not modified by exposure to these compounds (Table 2)

3.7- Plasma urea and protein levels

A significant interaction between HgCl$_2$ and (PhSe)$_2$ in plasma urea levels was observed ($F_{1,34}$=12,66, p<0.001). Mice exposed to HgCl$_2$ + (PhSe)$_2$ showed a higher increase in urea levels (~34%) when compared to those exposed to HgCl$_2$ (Table 3).

Two-way ANOVA yielded a significant interaction between HgCl$_2$ and (PhSe)$_2$ in plasma protein levels ($F_{1,25}$=139,17, p<0.001). Protein levels were reduced (~51%) in mice that received HgCl$_2$ + (PhSe)$_2$ when compared to those exposed to HgCl$_2$ (Table 3).

3.8- Urine glucose and protein levels

An increase in urine glucose and protein levels was observed in animals exposed to HgCl$_2$. This increase seems to be potentiated by (PhSe)$_2$ administration, since mice exposed to HgCl$_2$ + (PhSe)$_2$ showed higher urine glucose and protein levels than those exposed only to HgCl$_2$ (Table 4).

3.9- Hemoglobin, hematocrit and erythropoietin

Two-way ANOVA of hemoglobin levels data yielded a significant interaction between $HgCl_2$ and $(PhSe)_2$ ($F_{1,27}=24,54$, $p<0.001$). Animals exposed to $HgCl_2$ + $(PhSe)_2$ showed an increase (~48%) in hemoglobin levels when compared to those exposed to $HgCl_2$ (Table 5).

Two-way ANOVA of hematocrit data yielded a significant interaction between $HgCl_2$ and $(PhSe)_2$ ($F_{1,30}=33,70$, $p<0.001$). Mice exposed to $HgCl_2$ + $(PhSe)_2$ showed increased (~47%) hematocrit levels when compared to those exposed to $HgCl_2$ (Table 5). Erythropoietin content was not modified by exposure to $HgCl_2$ and $(PhSe)_2$ (Table 5).

3.10- Mercury concentration

Two-way ANOVA of renal mercury concentration data demonstrated a main effect of $HgCl_2$ exposure ($p<0.05$). The increased mercury concentration induced by $HgCl_2$ exposure was not significantly altered by $(PhSe)_2$ exposure (Figure 3A).

Two-way ANOVA of hepatic mercury concentration data demonstrated a main effect of $HgCl_2$ exposure ($p<0.05$). $(PhSe)_2$ administration did not significantly modify the increase in mercury concentration induced by $HgCl_2$ exposure (Figure 3B).

4- Discussion

Results from the present study indicate that $(PhSe)_2$ potentiated the damage induced by $HgCl_2$ exposure in mice. It is well known that inorganic mercury has a non-uniform distribution after absorption, being accumulated mainly in kidneys (Emanuelli et

al., 1996; Klaassen, 1996). Accordingly, kidney was the principal target tissue for mercury accumulation in the present study. In fact, we observed mercury levels about 4.5 times higher in kidney than in liver.

Data reported in the present study clearly show that $HgCl_2$ causes renal toxicity. In fact, mercury inhibited renal catalase activity and altered several biochemical parameters in plasma and urine of mice, which are indicators of renal dysfunction. It is interesting to note that mice exposed to $HgCl_2$ and $(PhSe)_2$ showed more pronounced alterations in renal tissue than those exposed only to $HgCl_2$. Consistent with the idea that $(PhSe)_2$ enhances the renal toxicity induced by $HgCl_2$, plasma urea and protein levels as well as urine glucose and protein concentrations were higher in mice exposed to the combined treatment ($HgCl_2$ + $(PhSe)_2$) than in those exposed only to $HgCl_2$. In addition, mice exposed to $HgCl_2$ + $(PhSe)_2$ showed decrease in plasma protein content. The elevated levels of plasma urea and urine glucose and protein associated with the reduction in plasma protein concentration reinforce the evidence of a renal toxicity induced by $HgCl_2$ + $(PhSe)_2$. Accordingly, proteinuria, glicosuria (Bernard and Lauwerys, 1990; Barbier et al., 2005) and uremia (Atessahin et al., 2003) have been reported as a result of proximal tubular damage and functional impairment of kidneys, respectively. It is important to point out that the parameter evaluated was urine total protein levels. Thus, we can not identify the specific site of injury (either glomerular or tubular segment or both). It is known that an increase in urine low molecular weight proteins indicates an inadequate absorption of these proteins by tubular cells, since, under physiological conditions, low molecular weight proteins (<40 kDa) filtered across the glomerular barrier are almost completely reabsorbed in the renal proximal tubule by receptor-mediated

endocytosis. In contrast, an increase in urine high molecular weight proteins indicates damage in glomerular cells, which leads to a release of high molecular weight proteins (>100 kDa) such as immunoglobulin G (150 kDa) (Christensen and Birn, 2002; Guignard and Santos, 2003).

Another evidence of the $HgCl_2$ + $(PhSe)_2$ toxic effect was the increased hemoglobin and hematocrit values in $HgCl_2$ + $(PhSe)_2$ exposed mice. The enhancement of these parameters could not be related to the erythropoietin synthesis, since concomitant administration of $HgCl_2$ + $(PhSe)_2$ did not alter the dosage of hormone. We can speculate that the reduced (~51%) plasma protein concentration induced by $HgCl_2$ + $(PhSe)_2$ could be involved in the increased hemoglobin and hematocrit values observed, as a result of a volemia reduction. This speculation is supported by the fact that plasma proteins are responsible for the volemia maintenance (Houpt and Yang, 1995). Therefore, a possible volemic shock could be, at least in part, the cause of mice death, previously reported by our research group (Brandão et al., 2006).

It is a well known that selenium (Nogueira et al., 2004) and mercury (Clarkson, 1997; Zalups and Barfuss, 1998) have a high affinity for SH groups of proteins and other endogenous biomolecules. Na^+,K^+-ATPase is a sulfhydryl-containing enzyme and, consequently, can be sensitive to oxidizing agents (Carfagna et al., 1996; Folmer et al., 2004). In the present study, renal Na^+,K^+-ATPase activity was not inhibited by administration of $HgCl_2$ or $(PhSe)_2$ in mice. The activity of Na^+,K^+-ATPase was only sensitive to the $HgCl_2$ + $(PhSe)_2$ association. It is likely that $HgCl_2$ and $(PhSe)_2$ form a complex or conjugate, which causes a Na^+,K^+-ATPase inhibition by oxidizing sulfhydryl groups, which are critical for the enzyme activity. Another data corroborating with this

idea is the observed inhibition of the δ-ALA-D activity induced by $HgCl_2$ + $(PhSe)_2$ association, in spite of a lack of enzyme inhibition in $HgCl_2$ exposed mice. δ-ALA-D is a sulfhydryl enzyme sensitive to heavy metals and selenium (Rocha et al., 1995; Farina et al., 2003b; Santos et al., 2005). In fact, Moller-Madsen and Danscher (1991) have reported that mercury binds to selenium to form mercury selenides (HgSe). The proposed mechanism to explain the oxidation of sulfhydryl groups by $HgCl_2$ and $(PhSe)_2$ association is described in **Scheme 1**.

Based on the fact that most of plasma mercury is bounded to serum proteins, such as albumin and selenoprotein P (SeP) (Lau and Sarkar, 1979) and that $(PhSe)_2$ and $HgCl_2$ complex has been reported in the literature (Isab et al., 2006), we propose the formation of a complex (**1**). The sulfhydryl groups of the enzyme substitute the chloro atoms in this molecule, via nucleophilic substitution-like reaction (Singh and Singh, 2002), to give the intermediary (**2**), causing the oxidation of the sulfhydryl groups of the enzyme (**3**) with delivery of the complex (**4**), which decomposes to diphenyl diselenide and elemental mercury. The substitution of the chloro atoms by the sulfur groups from the enzyme is supported by the greater nucleophilic character of sulfur than chloride and also by the fact that chlorine is one of the best living groups in this type of reaction.

Scheme 1 - Mechanism of sulfhydryl enzyme inhibition by $(PhSe)_2$ and $HgCl_2$ association
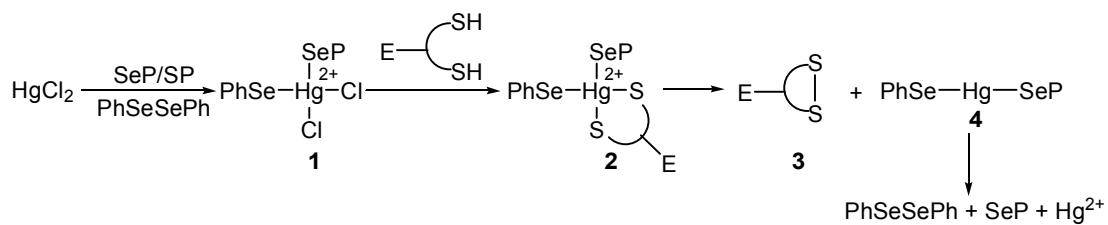
SeP selenium protein

SP sulfhydryl protein

ESH reduced sulfhydryl enzyme

ES-S oxidized sulfhydryl enzyme

Some evidences have been reported suggesting a synergistic (Brandão et al., 2005; Agarwal and Behari, 2007) and pro-oxidative (Huang et al., 1996; Nogueira et al., 2003a; Brandão et al., 2005) effects between mercury and selenium in mice. In the present study, we demonstrate that $HgCl_2$ + $(PhSe)_2$ exposure influenced, at least in part, the oxidative-antioxidative balance in mice kidney. In fact, mice exposed to $HgCl_2$ + $(PhSe)_2$ showed increased renal TBARS levels, which is indicator of lipid peroxidation (Huang et al., 1996). Therefore, lipid peroxidation, demonstrated by an increase in TBARS levels, could be an indicative of a renal toxicity induced by $HgCl_2$ + $(PhSe)_2$ association. Oxidative stress is characterized by a sharp increase in intracellular reactive oxygen species with the consequent release of metal ions from proteins. In many cases, this increase in pro-oxidant levels is mirrored by a decrease in the activity of antioxidant enzymes. In the present study, inhibition of renal catalase activity was evidenced in mice treated with $HgCl_2$ but $(PhSe)_2$ did not potentiate this effect.

GST, a phase II detoxifying enzyme, promotes the conjugation of reduced glutathione with a variety of reactive electrophilic compounds, resulting in the formation of less toxic substances that are easily excreted from the body (Chasseaud, 1979). Despite the important role of GST as a detoxifying enzyme, its antioxidant activity has also been reported (Casalino et al., 2004). Therefore, its inhibition can contribute to oxidative stress. In the present study, mice exposed to $HgCl_2$ + $(PhSe)_2$ showed an

inhibited renal GST activity. This inhibition emphasizes that $(PhSe)_2$ potentiated the oxidative damage induced by $HgCl_2$ in mice kidney. Regarding non-enzymatic antioxidant defenses, ascorbic acid levels were increased in mice treated with $HgCl_2$ + $(PhSe)_2$ association. The increase in ascorbic acid levels could be explained as a compensatory mechanism that counteracts the oxidative renal toxicity induced by the $HgCl_2$ + $(PhSe)_2$ exposure. Taking all the data together, the pro-oxidative effect of complex (**1**, Scheme 1) should be considered with caution, since other toxicity endpoints should be further analyzed.

In broad view, our data indicate that kidney and liver were important target tissues for $HgCl_2$ + $(PhSe)_2$ toxicity. Different from the results found in kidney, hepatic parameters were not modified by $HgCl_2$ exposure in mice. On the other hand, an oxidative toxicity induced by the concomitant administration of $HgCl_2$ + $(PhSe)_2$, although at lower extension, was also evident in the hepatic tissue. An increased hepatic TBARS level and an inhibition of catalase activity were observed in mice exposed to the $HgCl_2$ + $(PhSe)_2$ association. These findings are in accordance with those reported by other authors, demonstrating that the $HgCl_2$ and inorganic selenium association increased hepatic TBARS levels (Farina et al., 2003b).

In summary, our results demonstrated hepatic and mainly renal toxicity in mice exposed to the $HgCl_2$ + $(PhSe)_2$ association. One possible explanation for the $HgCl_2$ + $(PhSe)_2$ toxicity would be the important role of GSH in mercury uptake by kidney. In fact, increased NPSH (mainly GSH) levels in different tissues, including kidneys, has been reported after $(PhSe)_2$ administration in rodents (Barbosa et al. 2006; De Bem et al. 2007; Luchese et al. 2007). Therefore, the selenium effect on GSH levels could significantly alter the toxic effects of mercury. Conversely, data from mercury

determination demonstrated that $(PhSe)_2$ did not significantly modify the mercury levels in liver and kidney of mice exposed to $HgCl_2$, do not supporting the hypothesis raised above.

Although data showed that the combination of $HgCl_2$ and $(PhSe)_2$ affected a greater number of parameters in kidney when compared to those evaluated in the liver, it is not possible to identify the parameter(s) responsible(s) for the lethal effect observed. However, one can speculate that it is a renal toxicity that leads to mouse death and not extrarenal events, or a combination of both.

In conclusion, this study reports that the association of $HgCl_2$ and $(PhSe)_2$ potentiated damage caused by $HgCl_2$ in mice, affecting mainly the renal tissue. The association between $HgCl_2$ and $(PhSe)_2$ can present a hazard to population, since selenium compounds are often employed in organic synthesis and can be associated with mercury in an occupational environment.

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Legends

Figure 1. Effect of (PhSe)$_2$ on δ-ALA-D (A) Na$^+$, K$^+$- ATPase (B) and GST (C) activities in kidneys of mice exposed to HgCl$_2$. Data are reported as mean ± S.D. of six animals per group. (*) Denotes p<0.05 as compared to all the other groups (two-way ANOVA/Duncan). ($^\#$) Denotes p<0.05 as compared to the control group (two-way ANOVA/Duncan).

Figure 2. Effects of (PhSe)$_2$ on TBARS levels (A), CAT activity (B) and ascorbic acid levels (C) in kidneys of mice exposed to HgCl$_2$. Data are reported as mean ± S.D. of six animals per group. (*) Denotes p<0.05 as compared to all the other groups (two-way ANOVA/Duncan). ($^\#$) Denotes p<0.05 as compared to the control group (two-way ANOVA/Duncan).

Figure 3. Effect of (PhSe)$_2$ on mercury levels of kidney (A) and liver (B) from mice exposed to HgCl$_2$. Data are reported as mean ± S.D. of three animals per group. ($^\#$) Denotes p<0.05 as compared to the control group (two-way ANOVA/Duncan).

Tables

Table 1: Effect of (PhSe)$_2$ on δ-ALA-D and GST activities and TBARS levels in liver of mice exposed to HgCl$_2$

	δ-ALA-D	GST	TBARS
	(nmol PBG/mg/hour)	(µmol/min/mg prot)	(nmol MDA/mg prot)
Control	22.18 ± 3.91	0.54 ± 0.09	14.15 ± 2.61
HgCl$_2$	19.51 ± 4.84	0.63 ± 0.09	14.12 ± 2.48
(PhSe)$_2$	21.43 ± 2.78	0.52 ± 0.06	15.34 ± 1.94
HgCl$_2$ + (PhSe)$_2$	18.90 ± 2.38	0.61 ± 0.06	25.29 ± 3.91*

Data are reported as mean ± S.D. of six animals per group.
*Denotes $p < 0.05$ as compared to all the other groups (Two-way ANOVA/Duncan).

Table 2: Effect of (PhSe)$_2$ on CAT activity and ascorbic acid levels in liver of mice exposed to HgCl$_2$

	CAT	Ascorbic Acid
	(UI/mg prot)	(µg AA/g tissue)
Control	50.58 ± 8.37	400.99 ± 38.31
HgCl$_2$	45.78 ± 9.18	432.15 ± 31.14
(PhSe)$_2$	48.02 ± 10.78	441.43 ± 35.04
HgCl$_2$ + (PhSe)$_2$	35.12 ±7.40 *	409.78 ± 45.51

Data are reported as mean ± S.D. of six animals per group.
*Denotes $p < 0.05$ as compared to all the other groups (Two-way ANOVA/Duncan).

Table 3: Effect of $(PhSe)_2$ on plasma urea and protein levels of mice exposed to $HgCl_2$

	Plasma Urea (mg/dl)	Plasma Protein (g/dl)
Control	50.87 ± 2.42	20.71 ± 0.53
$HgCl_2$	175.57 ± 19.35 *	20.32 ± 0.64
$(PhSe)_2$	53.11 ± 6.71	20.94 ± 1.08
$HgCl_2$ + $(PhSe)_2$	235.36 ± 38.06 * #	10.66 ± 1.97 * #

Data are reported as mean ± S.D. of six animals per group.
* Denotes $p < 0.05$ as compared to the control and $(PhSe)_2$ groups (Two-way ANOVA/Duncan).
# Denotes $p < 0.05$ as compared to the $HgCl_2$ group (Two-way ANOVA/Duncan).

Table 4: Effect of $(PhSe)_2$ on urine protein and glucose levels of mice exposed to $HgCl_2$

	Urine protein	Urine glucose
Control	+; +; +; +; +	n.d.
$HgCl_2$	+; ++; ++; ++; +++	++; ++; ++; ++; ++
$(PhSe)_2$	+; +; +; +; ++	n.d.
$HgCl_2$ + $(PhSe)_2$	++; ++; +++; ++; +++	++; ++; +++; +++; +++

Data are reported of five animals per group.
Urine protein: (+) 30 mg/dl; (++) 100 mg/dL; (+++) 300 mg/dl.
Urine glucose: (++) 100 mg/dl; (+++) 250 mg/dl.
n.d. means undetectable urine glucose levels.

Table 5: Effect of $(PhSe)_2$ on hemoglobin, hematocrit and erythropoietin values of mice exposed to $HgCl_2$

	Hemoglobin (g/dl)	Hematocrit (%)	Erythropoietin (mU/ml)
Control	11.57 ± 0.63	39.25 ± 3.88	3.15 ± 1.34
$HgCl_2$	11.96 ± 1.86	40.78 ± 5.09	2.10 ± 0.14
$(PhSe)_2$	12.48 ± 1.34	40.75 ± 5.55	1.25 ± 0.35
$HgCl_2$ + $(PhSe)_2$	17.72 ± 1.28 *	60.11 ± 2.98 *	2.35 ± 1.76

Data are reported as mean ± S.D. of six animals per group.
* Denoted $p < 0.05$ as compared to all the other groups (Two-way ANOVA/Duncan).

Figures

Figure 1

A

B

Figure 2

A

B

C

Figure 3

A

B
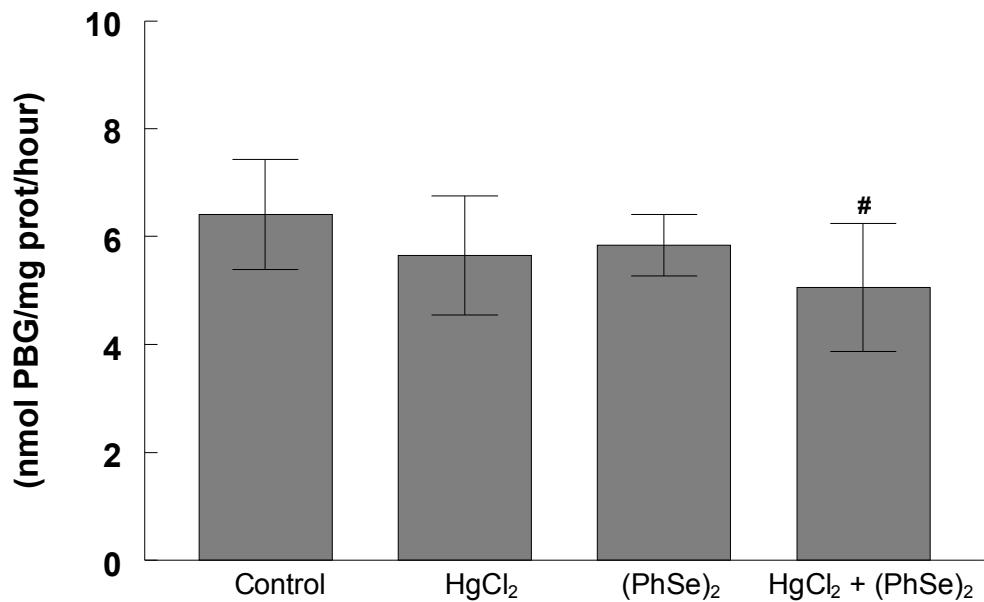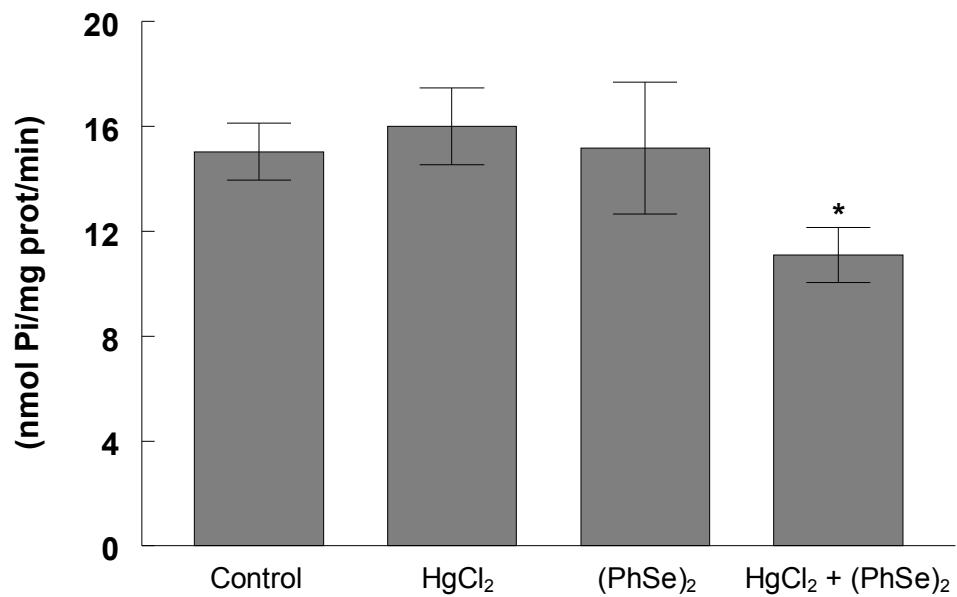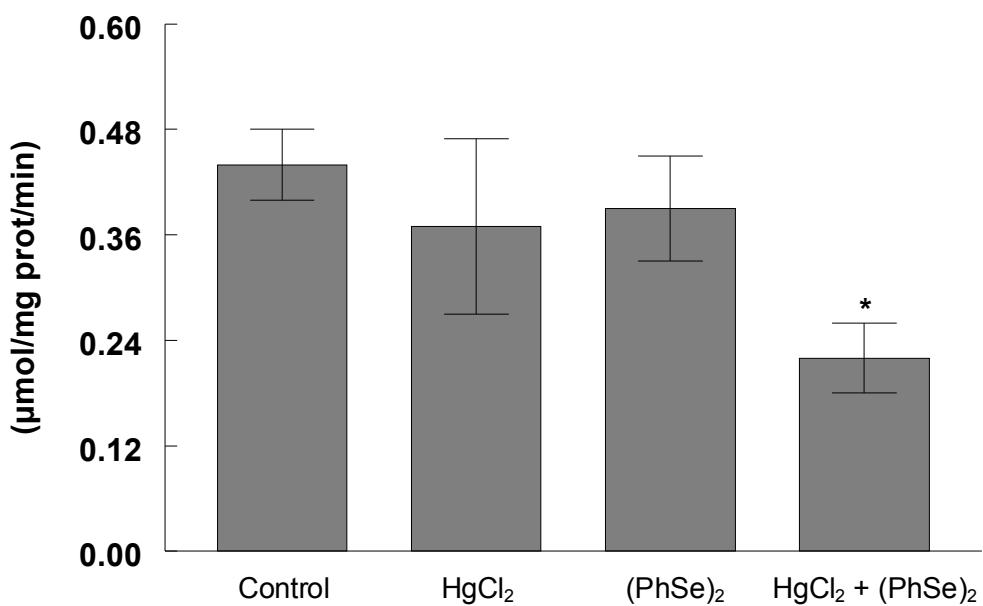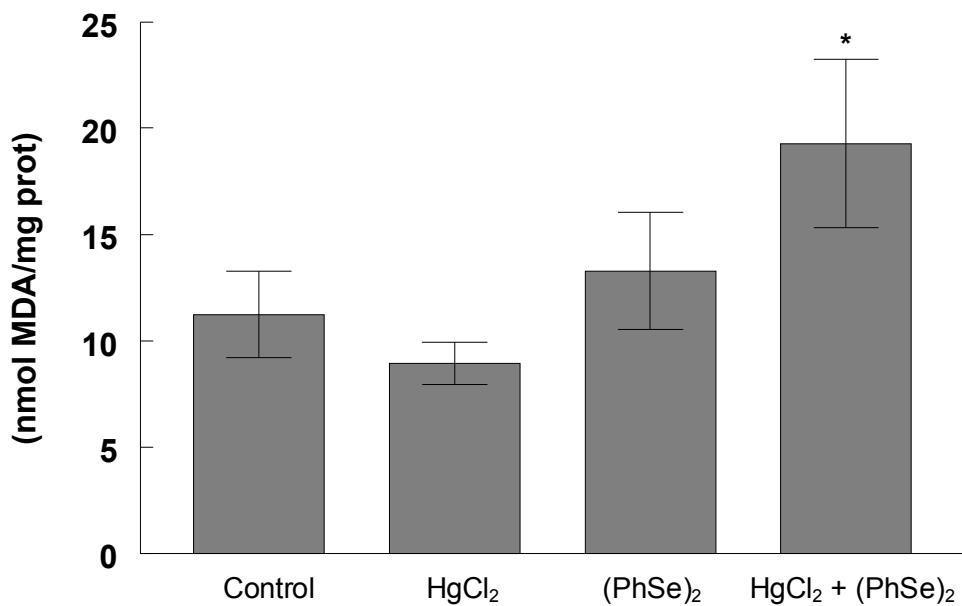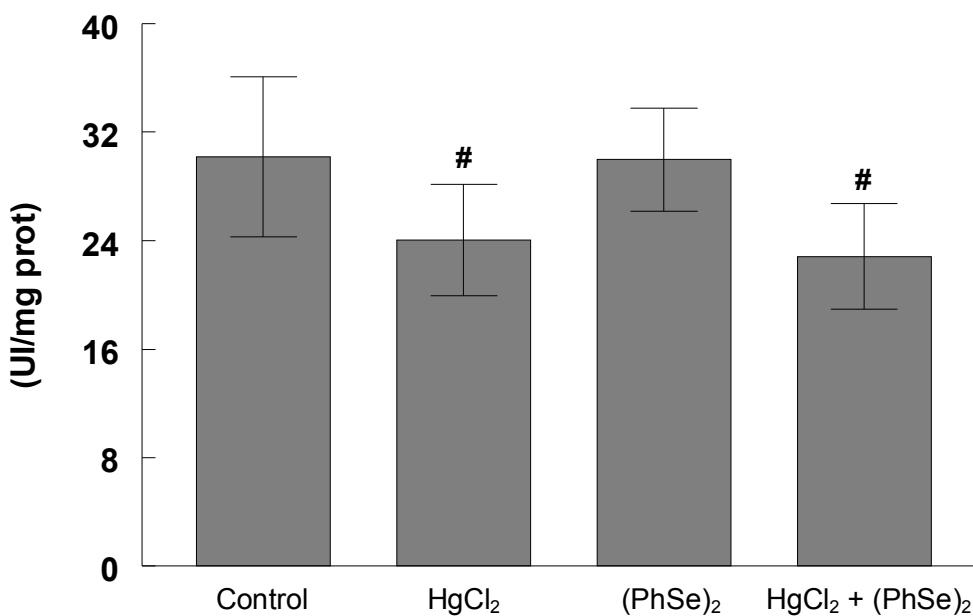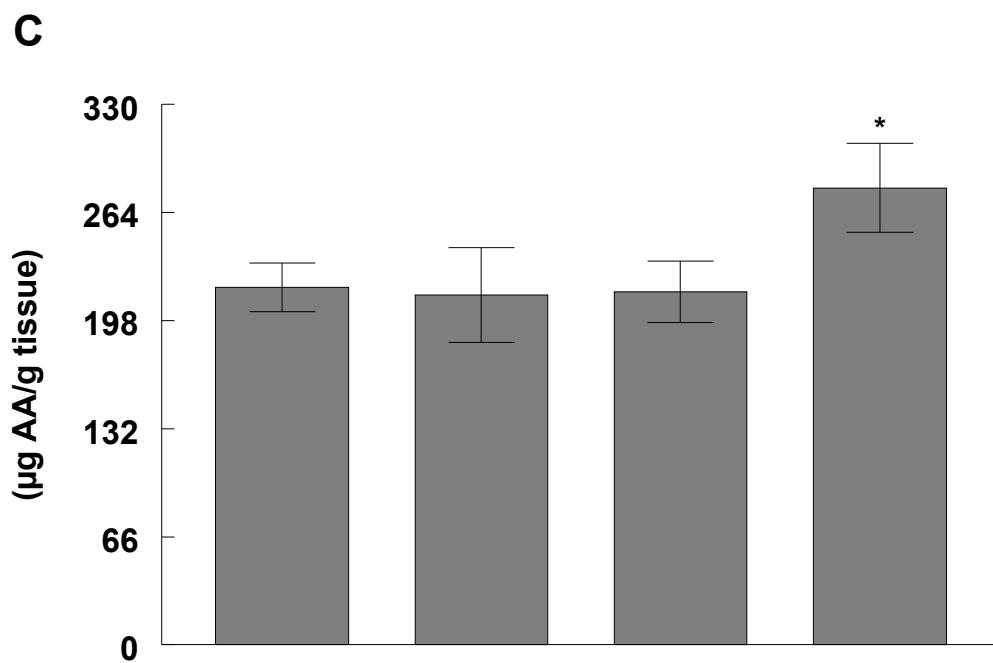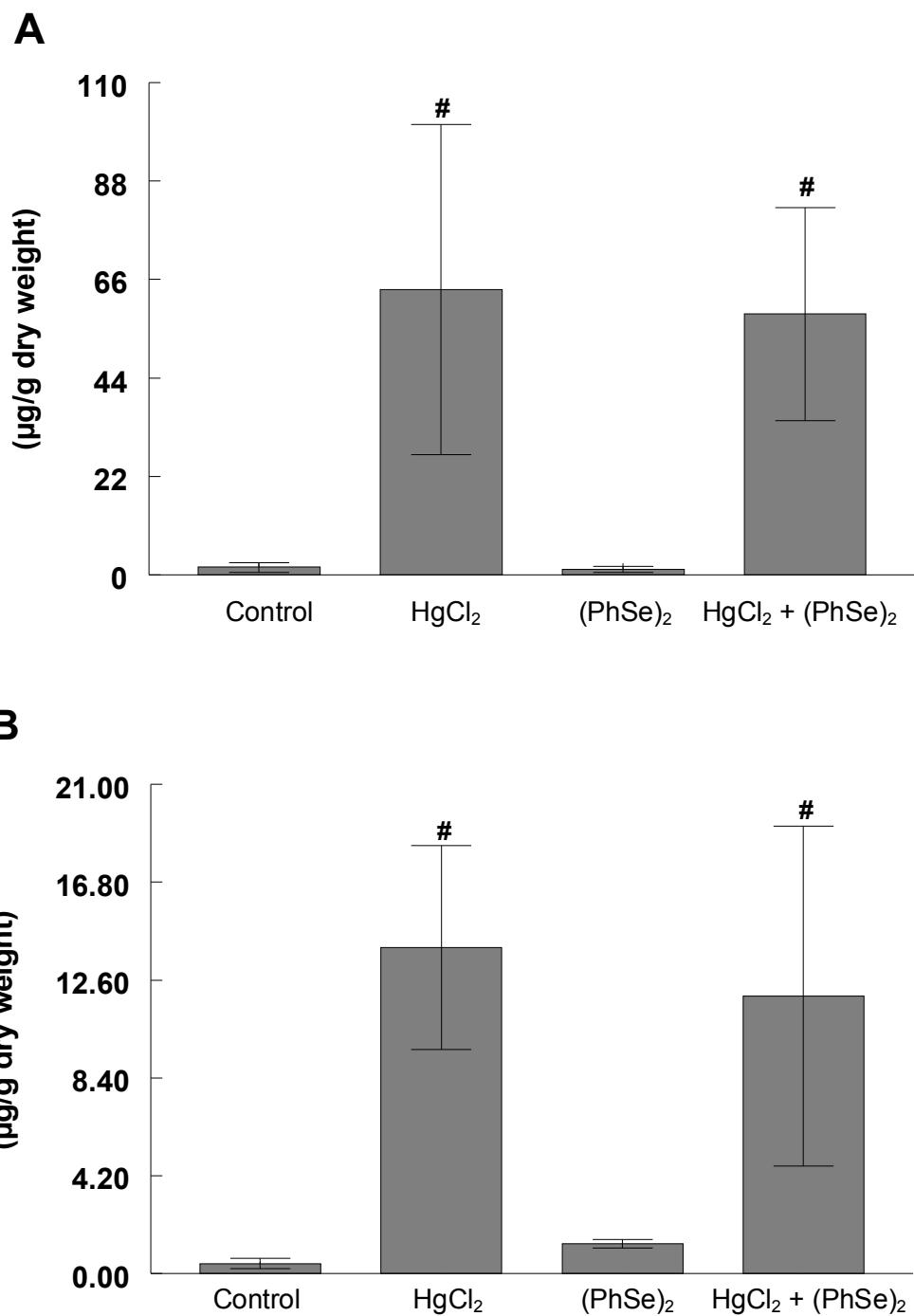
4.3. Disseleneto de difenila protege contra as alterações hematológicas e imunológicas induzidas pelo mercúrio em camundongos

4.3.1. Artigo 2

DIPHENYL DISELENDIDE PROTECTS AGAINST HEMATOLOGICAL AND IMMUNOLOGICAL ALTERATIONS INDUCED BY MERCURY IN MICE

Diphenyl Diselenide Protects Against Hematological and Immunological Alterations Induced by Mercury in Mice

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ABSTRACT: Mercury is a heavy metal that can cause a variety of toxic effects on the organism, such as hematological and immunological alterations. In the present investigation, deleterious effects of mercury-intoxication in mice and a possible protective effect of diphenyl diselenide (PhSe)$_2$ were studied. Male adult Swiss albino mice received daily a pretreatment with (PhSe)$_2$ (15.6 mg/kg, orally) for 1 week. After this week, mice received daily mercuric chloride (1 mg/kg, subcutaneously) for 2 weeks. A number of hematological (erythrocytes, leukocytes, platelets, hemoglobin, hematocrit, reticulocytes, and leukocytes differential) and immunological (immunoglobulin G and M plasma concentration) parameters were evaluated. Another biomarker of tissue damage, lactate dehydrogenase (LDH), was also determined. The results demonstrated that mercury exposure caused a reduction in the erythrocyte, hematocrit, hemoglobin, leukocyte, and platelet counts and an increase in the reticulocyte percentages. (PhSe)$_2$ was effective in protecting against the reduction in hematocrit, hemoglobin, and leukocyte levels. (PhSe)$_2$ ameliorated reticulocyte percentages increased by mercury. However, (PhSe)$_2$ was partially effective in preventing against the decrease in erythrocyte and platelet counts. Immunoglobulin G and M concentrations and LDH activity were increased by mercury exposure, and (PhSe)$_2$ was effective in protecting against these effects. In conclusion, (PhSe)$_2$ was effective in protecting against hematological and immunological alterations induced by mercury in mice. © 2008 Wiley Periodicals, Inc. J Biochem Mol Toxicol 22:311–319, 2008; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10:1002/jbt.20242

KEYWORDS: Mercury; Selenium; Diphenyl diselenide; Hematological; Immunological

INTRODUCTION

Inorganic mercury is widely used in certain types of batteries and dental amalgam fillings and continues to be an essential component of fluorescent light bulbs [1,2]. Also, a mixed burden of mercury species (mercury vapor, inorganic mercury, and methyl mercury) is assumed to be associated with human poisoning in gold mining areas [3].

Mercury has the potential to induce local and systemic hypersensitivity reactions in humans. The systemic reactions may include immune-mediated kidney diseases [4] and systemic autoimmune disease [5]. These reactions develop in only a fraction of similarly exposed individuals [6], indicating individual susceptibility to mercury-induced adverse immune reactions. It has been found that mercury can initiate hemolysis of red blood cells [7,8]. Hemolytic processes appear to be a consequence of metal ion inhibition of cytoplasmatic enzymes [9] and production of activated forms of oxygen by mercury–hemoglobin complexes [8]. In addition, mercury exposure can alter the leukocyte levels, since the immunological system is affected [10,11].

Mercury is a transition metal; it promotes the formation of reactive oxygen species (ROS) such as hydrogen peroxides [12]. Accordingly, mercury exposure has been demonstrated to induce lipid peroxidation [13]. Thus, it is believed that an antioxidant should be one of the important components of effective treatment for mercury poisoning [14].

Selenium is a structural component of several enzymes with physiological antioxidant properties, including glutathione peroxidase and thioredoxin reductase [15]. Besides, the ability of organic or inorganic

selenium to reduce mercury toxicity has been extensively investigated [16–19].

Diphenyl diselenide, $(PhSe)_2$, is an organoselenium compound that displays numerous pharmacological properties. Of particular importance are the hepatoprotective [20,21], antihyperglycemic [22], antiinflammatory and antinociceptive [23], antiulcer [24], neuroprotective [15], and antioxidant [25] properties, demonstrated in different experimental models. Recently, studies of our research group have also demonstrated that diphenyl diselenide protects against oxidative damage induced by cadmium in mice [26–28]. Therefore, the present study was aimed to investigate diphenyl diselenide effect on hematological and immunological alterations induced by mercury in mice.

MATERIALS AND METHODS

Chemicals

Diphenyl diselenide was prepared according to the literature method by Paulmier [29] and was dissolved in canola oil. Analysis of the ^1H NMR and ^{13}C NMR spectra showed that diphenyl diselenide presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of diphenyl diselenide (99.9%) was determined by GC/HPLC. $HgCl_2$ was obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Animals

Male adult Swiss albino mice (25–35 g) from our own breeding colony were used. The animals were kept in a separate animal room, on a 12-h light/dark cycle, at a room temperature of $22 \pm 2°C$ 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, the Federal University of Santa Maria, Brazil.

Experimental Procedure

To investigate the deleterious effects of mercury-intoxication in mice and a possible protective effect of pretreatment with diphenyl diselenide, mice received daily, by gavage, diphenyl diselenide (15.6 mg/kg, dissolved in canola oil) (groups 3 and 4) for 1 week. After the first week, mice received daily mercuric chloride by subcutaneous route (1 mg/kg, dissolved in 0.9% NaCl) (groups 2 and 4) for 2 weeks. The exposure regimen and the dose of mercuric chloride were selected based on Booker and White's study [30]. This study showed immunological alterations in mice exposed to

mercuric chloride. The dose of diphenyl diselenide was chosen based on LD_{50} study, which demonstrated that 15.6 mg/kg did not cause toxicity in mice [31]. Control groups received only vehicle (canola oil, 2.5 mL/kg and saline, 10 mL/kg) (group 1).

The protocol of mice treatments is given as follows:

Group 1: Canola oil (2.5 mL/kg, p.o.) plus 0.9% NaCl (10 mL/kg, s.c.);

Group 2: Canola oil (2.5 mL/kg, p.o.) plus $HgCl_2$ (1 mg/kg, s.c.);

Group 3: Diphenyl diselenide (15.6 mg/kg, p.o.) plus 0.9% NaCl (10 mL/kg, s.c.);

Group 4: Diphenyl diselenide (15.6 mg/kg, p.o.) plus $HgCl_2$ (1 mg/kg, s.c.).

After the third week of experiment, mice were anesthetized (ketamine chloridate and xylazine, 5:1; 0.1 mL/100 g) for blood collection by heart puncture. An aliquot of 500 μL of total blood was separated on vacuum tubes containing EDTA K_3 and used for hematological assays, such as erythrocyte, leukocyte, and platelet counts, hemoglobin content, hematocrit, and percentage of reticulocytes, lymphocytes, monocytes, and neutrophils. Plasma was obtained by centrifugation at $2000 \times g$ for 10 min (hemolyzed plasma was discarded) and used for biochemical (lactate dehydrogenase) and immunological assays (IgG and IgM).

Hematological Evaluation

Hematological parameters were evaluated using a Pentra 120 Retic-Count Analyzer™ (HORIBA ABX Brasil, São Paulo, Brazil). The methods used by this equipment are flow cytometry, impedance, photometry, and cytochemistry. The principle of flow cytometry and impedance is based on the method of analysis of anticoagulated blood samples (3 μL) that pass through a particular channel where the cells are detected and separated according to weight, size and morphology, using a pulse of electrons to detect the parameters that are converted in the required values by specific software. The photometry is used for detection of values of absorbance of hemolysate blood samples and determines only the levels of hemoglobin. The cytochemistry uses different reagents that mark the different cells in accordance with the morphology. These marked cells are detected by a specific laser and are separated according to the groups.

Erythrogenesis Profile

The evaluation of bone marrow viability was investigated by reticulocyte counts of total blood, using a Pentra 120 Retic-Count Analyzer™ (HORIBA ABX

Brasil). The peripheral blood smears were analyzed by optical microscopy to confirm the results. Reticulocytes were counted in anticoagulated blood samples using the methylene blue staining. This counting was accomplished by visual observation of 2000 cells.

Cellular Damage Marker

Lactate dehydrogenase (LDH) activity was monitored spectrophotometrically by the rate of increase in absorbance at 340 nm at 30°C resulting from the formation of NADH [32]. The assay medium contained 21 mM of glycine-KOH buffer (pH 10), 0.25 mM of NAD^+, and 25 μL of plasma. The mixture was preincubated for 3 min, and the reaction was started by adding neutralized lactic acid (pH 6.8) to provide a final concentration of 50 mM. The reaction was linear for up to 2 min.

Immunological Parameters

Plasma levels of IgG and IgM were measured using an immunoturbidimetry assay (Turbitimer-Dade Behring™). Briefly, when a diluted antigen solution is mixed with a corresponding antibody (anti-IgG and anti-IgM antibodies), the reaction between the antigen and the antibody results in the formation of immune complexes. The solution became turbid and was measured by optical detection. Antibodies were purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL).

Morphological Aspects

To evaluate morphological aspects, peripheral blood smears were stained with reagent for microscopy (Bioclin, Paraná, Brazil) and analyzed by optical microscopy to confirm the hematological counts. Red and white blood cells images were obtained using a color charge-coupled device camera coupled to an Olympus BX-51 light microscope.

Statistical Analysis

The results are presented as means ± SD. Statistical analysis was performed using a two-way analysis of variance (ANOVA), followed by Duncan's multiple range test when appropriate. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Hematological Evaluation

Two-way ANOVA showed a significant interaction between HgCl₂ and (PhSe)₂ in erythrocyte levels
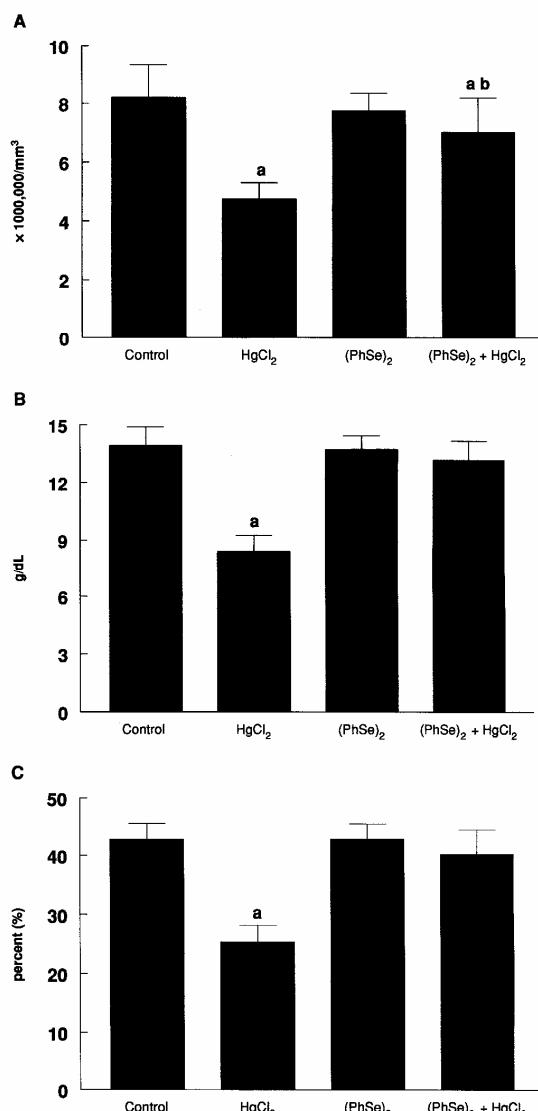
FIGURE 1. Effect of diphenyl diselenide, (PhSe)₂, on erythrocytes (1A) and hemoglobin (1B) levels and on the hematocrit (1C) of mice exposed to HgCl₂. Data are reported as mean ± SD of six to eight animals per group. [a]Denoted $p < 0.001$ as compared to the control group (two-way ANOVA/Duncan). [b]Denoted $p < 0.001$ as compared to the HgCl₂ group (two-way ANOVA/Duncan).

$(F_{1,31} = 17.21, p < 0.001)$, hemoglobin concentration $(F_{1,38} = 77.7, p < 0.001)$, and hematocrit $(F_{1,36} = 57.26, p < 0.001)$ (Figure 1). In fact, these results indicated that HgCl₂ exposure reduced erythrocyte levels (42%) (Figure 1A), hemoglobin concentration (39%)
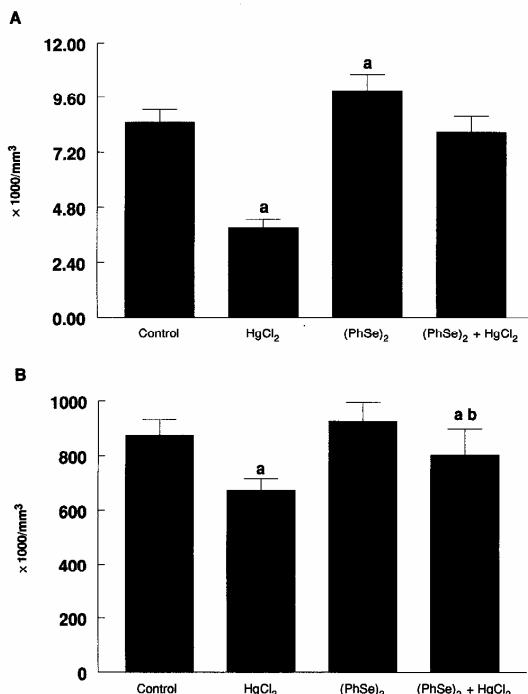
**FIGURE 2.** Effect of diphenyl diselenide, (PhSe)$_2$, on leukocyte (2A) and platelet (2B) levels of mice exposed to HgCl$_2$. Data are reported as mean ± SD of six to eight animals per group. [a]Denoted $p <$ 0.001 as compared to the control group (two-way ANOVA/Duncan). [b]Denoted $p <$ 0.001 as compared to the HgCl$_2$ group (two-way ANOVA/Duncan).

(Figure 1B), and hematocrit (40%) (Figure 1C). The (PhSe)$_2$ pretreatment was effective in protecting against the reduction of hematocrit and hemoglobin levels induced by HgCl$_2$. The reduction of erythrocyte concentration induced by mercury was partially ameliorated by pretreatment with (PhSe)$_2$.

A significant interaction between HgCl$_2$ and (PhSe)$_2$ in leukocyte levels was observed ($F_{1,30} = 44.63$, $p < 0.001$) (Figure 2A). The results indicated that mice exposed to HgCl$_2$ had a decrease in leukocyte levels (54%), and the pretreatment with (PhSe)$_2$ was effective in protecting against this reduction. The results also demonstrated that mice exposed only to (PhSe)$_2$ had an increase in leukocyte levels (16%).

Two-way ANOVA of platelet concentration did not reveal interaction between HgCl$_2$ and (PhSe)$_2$ ($F_{1,30} = 3.0$, $p < 0.094$) (Figure 2B). A main effect of HgCl$_2$ and (PhSe)$_2$ ($p < 0.001$) on the platelet levels was observed. Mice exposed to HgCl$_2$ presented a decrease in platelet counts (23%), and (PhSe)$_2$ was partially able in protecting against this reduction.

TABLE 1. Effect of Diphenyl Diselenide, (PhSe)$_2$, on Percentage of Lymphocytes, Monocytes, and Neutrophils of Mice Exposed to Mercury

	Lymphocytes (%)	Monocytes (%)	Neutrophils (%)
Control	83.52 ± 6.25	4.15 ± 1.15	8.0 ± 1.42
HgCl$_2$	82.37 ± 8.32	2.99 ± 0.67	11.55 ± 3.42[a]
(PhSe)$_2$	82.0 ± 5.78	5.71 ± 2.02[a]	9.2 ± 1.17
(PhSe)$_2$ + HgCl$_2$	83.2 ± 5.92	4.61 ± 1.52	9.63 ± 1.09

[a]Denoted $p < 0.05$ as compared to the control group (two-way ANOVA/Duncan).

Two-way ANOVA of lymphocyte, monocyte, and neutrophil percentages did not show a significant interaction between HgCl$_2$ and (PhSe)$_2$ ($F_{1,35} = 0.26$, $p < 0.611$; $F_{1,29} = 0.0$, $p < 0.948$; $F_{1,25} = 3.60$, $p < 0.069$, respectively) (Table 1). Two-way ANOVA of monocyte percentages showed a main effect of (PhSe)$_2$ ($p < 0.05$), demonstrated by the increase in the monocyte percentages (37%) in mice treated with (PhSe)$_2$. Regarding neutrophils, two-way ANOVA revealed a main effect of HgCl$_2$ ($p < 0.05$), evidenced by the augmentation in neutrophil percentages (44%) in mice exposed to HgCl$_2$.

Erythrogenesis Profile

The results demonstrated a significant interaction between HgCl$_2$ and (PhSe)$_2$ in reticulocyte percentage ($F_{1,40} = 59.81$, $p < 0.001$) (Figure 3). (PhSe)$_2$ pretreatment was effective in protecting against the increase in reticulocyte percentages induced by mercury.

Cellular Damage Marker

Two-way ANOVA revealed significant interaction between HgCl$_2$ and (PhSe)$_2$ in LDH activity ($F_{1,36} =$
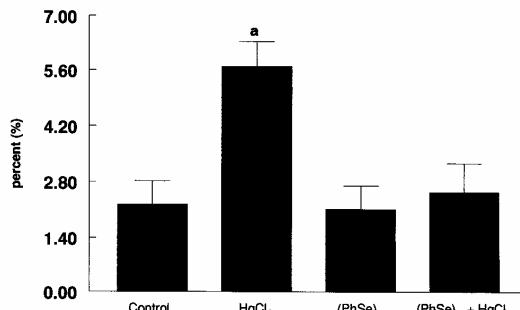

FIGURE 3. Effect of diphenyl diselenide, (PhSe)$_2$, on the reticulocyte percentages of mice exposed to HgCl$_2$. Data are reported as mean ± SD of six to eight animals per group. [a]Denoted $p < 0.001$ as compared to the control group (two-way ANOVA/Duncan).
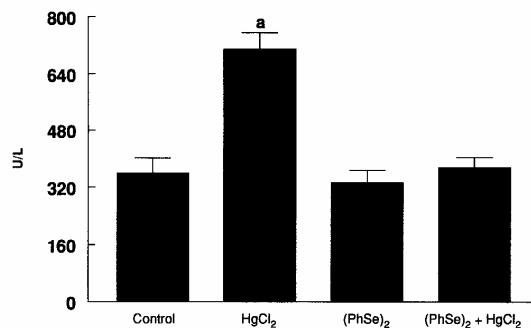
FIGURE 4. Effect of diphenyl diselenide, (PhSe)$_2$, on LDH activity of mice exposed to HgCl$_2$. Data are reported as mean ± SD of six to eight animals per group. [a]Denoted $p < 0.001$ as compared to the control group (two-way ANOVA/Duncan).

153.61, $p < 0.001$). In fact, mercury exposure increased LDH activity (about 2.0-fold) (Figure 4) and (PhSe)$_2$ pretreatment was effective in protecting against this alteration.

Immunological Parameters

We observed a significant interaction between HgCl$_2$ and (PhSe)$_2$ in IgG concentration ($F_{1,39} = 155.39$, $p < 0.001$) (Figure 5A). Mice exposed to mercury had an increase in IgG levels (about 2.6-fold), and (PhSe)$_2$ was able to protect against this effect.

Two-way ANOVA demonstrated significant interaction between HgCl$_2$ and (PhSe)$_2$ in IgM concentration ($F_{1,30} = 272.96$, $p < 0.001$) (Figure 5B). (PhSe)$_2$ was able to protect the increase in IgM levels (about 4.0-fold) induced by HgCl$_2$.

Morphological Evaluation

Morphological analysis is in accordance with the immunological and hematological data presented in this study. Peripheral blood smears demonstrated normal distribution and morphology of red cells (Figure 6A). HgCl$_2$ exposure induced the presence of polychromatophils (reticulocytes in peripheral blood) (Figure 6C, white arrow), platelet aggregation (Figure 6C, black arrow), and the increase of the spaces between red cells (Figure 6D). It is interesting to observe that mice treated with diphenyl diselenide had morphological analysis preserved. In fact, diphenyl diselenide was able to prevent morphological status of red cells, white cells, spaces between cells, and platelet aggregation (Figure 6E). Diphenyl diselenide
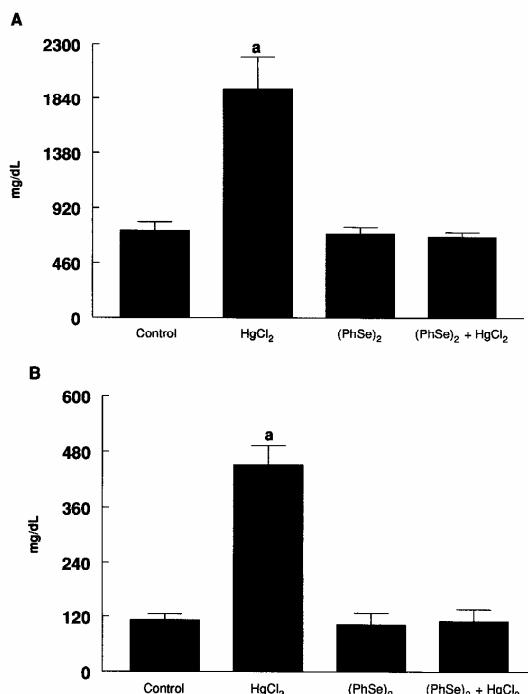
FIGURE 5. Effect of diphenyl diselenide, (PhSe)$_2$, on IgG (A) and IgM (B) levels of mice exposed to HgCl$_2$. Data are reported as mean ± SD of six to eight animals per group. [a]Denoted $p < 0.001$ as compared to the control group (two-way ANOVA/Duncan).

alone did not modify cellular conformation and distribution (Figure 6B).

DISCUSSION

It is well explored that the industrial use of mercury can cause general toxic effects on human and animal systems [33,34]. The aim of this study was to evaluate diphenyl diselenide effect on hematological and immunological alterations induced by mercury in mice. Oral treatment with diphenyl diselenide was effective in protecting against the immunological and hematological alterations induced by mercury in mice.

Selenium is a component of several enzymes with antioxidant properties, including glutathione peroxidase and thioredoxin reductase [15]. In addition, it has been well documented that mercury and selenium interact in the body of mammals, and the coadministration of both reduces the toxicity of each other [35]. However, studies reporting beneficial effects of (PhSe)$_2$ on hematological and immunological disorders induced
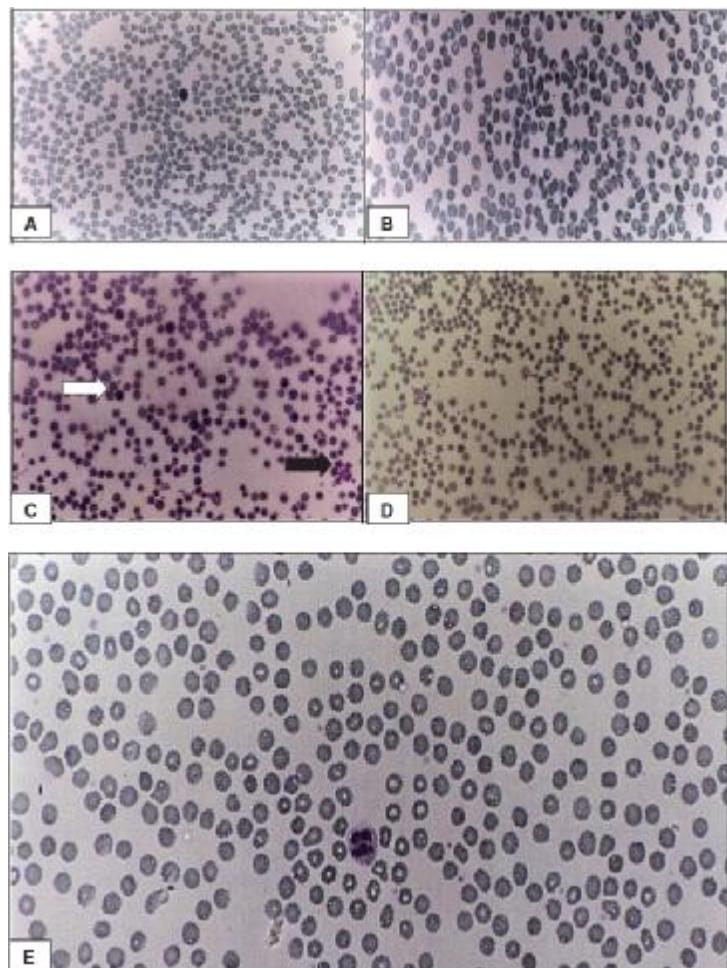
FIGURE 6. Effect of diphenyl diselenide, $(PhSe)_2$, on peripheral blood smears of mice exposed to $HgCl_2$. (A) control, (B) $(PhSe)_2$, (C) $HgCl_2$ treated mice with polychromatophils (white arrow) and platelet aggregates (black arrow), (D) $HgCl_2$ treated mice with a large space between erythrocytes, (E) $(PhSe)_2$ plus $HgCl_2$ treated mice. ($\times 400$).

by $HgCl_2$ do not appear in the literature. A number of studies have demonstrated that diphenyl diselenide displays pharmacological properties, such as antiulcer [24], antihyperglycemic [22], and hepatoprotector [20,21].

Hematological evaluation of mice treated with $HgCl_2$ revealed the appearance of anemia, demonstrated by a decrease in erythrocyte number, hemoglobin, and hematocrit (Figure 1). $(PhSe)_2$ was capable of protecting against the reduction in hematocrit and hemoglobin levels. However, the reduction in erythrocyte levels by $HgCl_2$ was partially prevented by $(PhSe)_2$. In fact, these results are confirmed by morphological aspects (see Figures 6C and 6D). Zolla and

collaborators [8] have reported that mercury exposure induces hemolysis and autoimmune response, and this effect seems to be related to prooxidative effects induced by mercury. Therefore, the hemolytic process appears to be a consequence of metal ion inhibition of antioxidant cytoplasmatic enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase [9]. Otherwise, nonenzymatic defenses can also be affected, since mercury decreases glutathione levels in erythrocytes [9]. Another possible mechanism of anemia induced by mercury is the production of activated forms of oxygen by mercury–hemoglobin complexes [8] and alterations in hemoglobin structure (blocking –SH groups of globin chains) [36]. Since the anemia

102

induced by HgCl$_2$ can be explained by prooxidant effects of this heavy metal, the protective effect of (PhSe)$_2$ can be related to its antioxidant property [15].

Leukocyte levels, a marker of cellular defense, were reduced after exposure to HgCl$_2$. It is known that inorganic mercury and its salts induce abnormal responses in the immune system of both susceptible rodent strains (rat and mouse) and humans [37,38]. In accordance, Girardi et al. [10] have demonstrated that isolated renal glomeruli from mercuric chloride treated rats showed enlarged fibronectin, lipid deposits, and enhanced leukocyte infiltration. Therefore, we speculate that the reduction in leukocyte levels in the blood stream can be explained by cellular migration to damage sites, since these cells have an important function of defense, or hypothetically by the loss of blood cells that may reduce their levels. (PhSe)$_2$ was effective in protecting against the reduction in the leukocyte levels induced by mercury. Moreover, mice treated only with (PhSe)$_2$ had an increase in the leukocyte levels. These data are in accordance with selenium immunomodulatory properties, which are mediated through improved leukocyte function [39]. Greenman et al. [40] have reported that short-term selenium supplementation increased polymorphonuclear leukocyte phagocytosis.

In this study, HgCl$_2$ induced an increase in the percentage of neutrophils, which may indicate the activity of the first line of defense against the oxidative damage, and (PhSe)$_2$ was effective in protecting against this increase. According to this, several studies have demonstrated that HgCl$_2$ could reduce polymorphonuclear migration [41,42]. Hypothetically, this reduction in migration may be related to the increase in percentage of neutrophils in the blood stream. Interestingly, (PhSe)$_2$, when administered alone, induced an increase in monocyte levels, which probably reflects one of the mechanisms by which this compound exerted its protective effect. Monocytes are the most effective second-line defense against cellular damage, cleaning the site of damage by phagocytic properties.

Kumar has demonstrated that heavy metals, such as mercury and arsenic, increase the platelet aggregation, because these metals stimulate phosphodiesterase activity and inhibit adenylate cyclase, reducing cyclic adenosine monophosphate level. The platelet aggregation was found to increase in mice exposed to HgCl$_2$ (see Figure 6C, black arrow), which can cause a decrease in platelets counts. (PhSe)$_2$ was partially effective in protecting against the reduction in platelet levels.

Another finding of this study was the increase in reticulocyte percentages induced by HgCl$_2$. Reticulocytes are cells in the transitional stage when acidophilic erythroblasts are converted to mature erythrocytes. Normal blood cells contain about 1% of reticulocytes. However, in pathological conditions such as hemolytic

and blood-loss anemia, the number of reticulocytes is increased as a response of bone marrow to anemia [44]. We speculate that the protocol of mercury exposure used in this study did not interfere with bone marrow cellularity, since the increase of reticulocyte levels was observed in peripheral blood (Figure 6C, white arrow). Mice exposed to HgCl$_2$ and treated with (PhSe)$_2$ had normal reticulocyte percentages, explained by the fact that these animals did not have anemia.

There are three major possible causes of anemia: blood loss, inadequate production of red blood cells, and hemolysis. Mice were not bled prior to sacrifice; therefore, blood loss was not the cause of anemia. HgCl$_2$ exposure increases the reticulocyte percentages in the peripheral blood, which tends to include this effect to massive peripheral erythrocytic destruction and respective activation of bone marrow production of new cells, which is corroborated by the red aspect of plasma observed macroscopically (data not shown). This makes hemolysis the most likely cause of anemia.

LDH is a parameter widely used in toxicology and in clinical chemistry to diagnose cell, tissue, and organ damage [45]. Hemolysis and any tissue damage were evidenced by an increase of LDH activity in mice exposed to HgCl$_2$. (PhSe)$_2$ was able to reduce LDH activity altered by HgCl$_2$ exposure. LDH is an important glycolytic enzyme that is present in virtually all tissues [46]. Therefore, the hemolysis found in mice exposed to HgCl$_2$ is in agreement with the increase in LDH activity.

The involvement of the immunological system in HgCl$_2$ exposure was confirmed by the increase in IgG and IgM concentrations. These results are in accordance with Bigazzi [47], who demonstrated that the repeated administration of mercury into rats induces the production of antibodies. In addition, several studies have reported that increased immunoglobulin levels were found in workers exposed to mercury [48–50]. Another interesting study demonstrated that genetically susceptible mice, treated with mercury, presented an increased number of immunoglobulin-secreting cells [51] and hyperimmunoglobulinemia [52]. The immunoglobulins are antibodies produced by lymphocyte B and the increase in the immunoglobulin level is a response to some pathology, such as infections, hepatic diseases, pulmonary problems, and autoimmune alterations [53]. In fact, it is well known that mercury induces a polyclonal activation of B-cells in rats [54] and this immunological activation may induce systemic autoimmune disease [5]. Our results indicate that the increase in IgG and IgM levels is, probably, a response of the immunological system to mercury toxicity, since these immunoglobulins are released in all acute phase of damage. The immunological alterations induced by HgCl$_2$ were abolished by

(PhSe)$_2$, confirming the immunomodulatory effects of this organoselenium compound.

Our results indicated that (PhSe)$_2$ protects against all morphological alterations induced by HgCl$_2$ (Figure 6E). The protective effect of (PhSe)$_2$ against metals toxicity has been demonstrated [26–28].

There are two possible mechanisms proposed to explain (PhSe)$_2$ protection against the toxicity induced by HgCl$_2$: (a) the antioxidant action of this compound [15] and (b) the formation of an inactive complex between mercury and selenium, or mercury, selenium, and seleneprotein P, reducing the availability of mercury [55].

It is important to point out that the dose of HgCl$_2$ used in this study is relevant in terms of potential human exposure. This assertion is based on the following considerations: (i) the maximum concentration of mercury permitted in human blood is 15 µg/L [56]; (ii) Jin et al. [57] showed that mice exposed to subcutaneous injection of 1 mg/kg HgCl$_2$, three times per week for 3 weeks, presented about 200 µg/L of mercury in the blood. Although in the present study we did not evaluate mercury concentration in the blood of mice, the exposure regimen used by Jin and collaborators [57] is very similar to that used by us in the present study; and (iii) WHO [58] reported that humans commonly exposed to inorganic mercury presented a range of 100–200 µg/L of mercury in blood.

In conclusion, the current study demonstrates for the first time that (PhSe)$_2$ protected against the hematological and immunological alterations induced by HgCl$_2$ in mice. The possible mechanisms involved in (PhSe)$_2$ effect are its antioxidant activity and/or the formation of inactive complexes. (PhSe)$_2$ can be a promising agent used in the prevention of mercury intoxication in humans, but more studies are required to elucidate the exact mechanism by which this organoselenium compound exerts its protective effects.

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4.4. Concomitante administração de 2,3-dimercapto-1-propanosulfonato (DMPS) e disseleneto de difenila reduz a efetividade do DMPS em restaurar o dano induzido pelo cloreto de mercúrio em camundongos

4.4.1. Manuscrito 2

CONCOMITANT ADMINISTRATION OF SODIUM 2,3-DIMERCAPTO-1-PROPANESULPHONATE (DMPS) AND DIPHENYL DISELENIDE REDUCES EFFECTIVENESS OF DMPS IN RESTORING DAMAGE INDUCED BY MERCURIC CHLORIDE IN MICE

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Concomitant Administration of Sodium 2,3-Dimercapto-1-Propanesulphonate (DMPS) and Diphenyl Diselenide Reduces Effectiveness of DMPS in Restoring Damage Induced by Mercuric Chloride in Mice

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Abstract

This study was designed to determine the possible restorative effect of combined therapy with diphenyl diselenide (PhSe)$_2$ and sodium 2,3-dimercapto-propane-1-sulphonate (DMPS) against renal and hematological damage induced by mercury (HgCl$_2$) in mice. Male adult Swiss albino mice were exposed to HgCl$_2$ (1 mg/kg, subcutaneously) for two weeks. After that, mice received by gavage (PhSe)$_2$ (15.6 mg/kg) and DMPS (12.6 mg/kg), individually or combined, for one week. Hematological parameters (erythrocytes, leukocytes and platelets) were evaluated in blood of mice. Thiobarbituric acid-reactive substances (TBARS), ascorbic acid and mercury levels and glutathione S-transferase (GST) and catalase (CAT) activities were investigated in kidney of mice. In addition, plasma was used to billirubin, uric acid, urea and creatinine levels as well as lactate dehydrogenase (LDH) activity determinations. (PhSe)$_2$ and DMPS, administered individually, were effective in restoring LDH activity, the levels of TBARS, uric acid, urea and creatinine, erythrocytes, hemoglobin and hematocrit modified by HgCl$_2$ exposure. Leukocyte, monocyte, neuthrophil, limphocyte, platelet counts and total and indirect bilirubin levels modified by HgCl$_2$ exposure were also restored by (PhSe)$_2$ or DMPS. DMPS restored the increase in mercury levels induced by HgCl$_2$. Concomitant administration of (PhSe)$_2$ and DMPS reduced the effectiveness of DMPS in restoring damage induced by HgCl$_2$ exposure in mice. In conclusion, combined therapy with (PhSe)$_2$ and DMPS was less effective than isolated therapies in restoring renal and hematological damage induced by HgCl$_2$. Therefore, the association of (PhSe)$_2$ and DMPS seems to be not a good choice to counteract the damage induced by HgCl$_2$ in mice.

Key-Words: mercury, selenium, diphenyl diselenide, kidney, hematology.

1. Introduction

Humans may be exposed to mercury in its various forms, including Hg^{2+}, from breathing contaminated air, ingesting contaminated water and food, by having dental and medical treatments (ATSDR, 1999) and by using cosmetic and household products (Ozuah, 2000). Thus, besides being an occupational concern, due to the widespread use of mercurials in various industrial processes, Hg^{2+} is a public health concern as well (Lash et al., 2007). Various mechanisms, including lipid peroxidation, have been proposed for the biological toxicity of mercury (Mahboob et al., 2001; Hussain et al., 1999; Kim and Sharma, 2003). Over the last decade evidence has accumulated for a role of reactive oxygen metabolites as a mediator of tissue injury in several models of toxicity including $HgCl_2$ (Sener et al., 2007). The kidneys are the primary organs that accumulate Hg^{2+} and exhibit toxic effects after in vivo exposure to this element (Sharma et al., 2005). The cellular content of thiols, particularly that of glutathione (GSH), can modulate the intracellular uptake, cellular accumulation, and toxicity of Hg^{2+} in the renal proximal tube (Zalups, 1997; Lash et al., 1998).

A method for the detoxification of mercury that is widely recommended is its transformation into a chelate complex (Jones, 1994), which has a water solubility greater than that of the mercury and therefore is readily excreted in the urine (Vamnes et al., 2003). In this manner, body levels of mercury are reduced to a less dangerous concentration. Meso-2,3-dimercaptosuccinic acid (DMSA) and sodium 2,3-dimercapto-propane-1-sulphonate (DMPS) are dithiol chelating agents used in the treatment of mercury toxicity (Aposhian et al., 1995; Rooney, 2007). DMPS is not currently approved by the FDA for any clinical use, although it is being used to treat mercury toxicity in an off-label capacity (Risher and Amler, 2005).

Selenium is known to affect the distribution of mercury and also to reduce toxicity induced by mercury in experimental animals (Goyer et al., 1995). However, it has been observed in rats that simultaneous administration of selenium with a chelating agent (DMPS or DMSA) leads to reduced effectiveness of the chelators (Juresa et al., 2005). It is noteworthy that the form of selenium intake is also relevant (Xia et al., 2005). Numerous studies have revealed the ability of organic or inorganic selenium compounds to reduce mercury toxicity (Sasakura and Suzuki 1998; Farina et al., 2003; Perottoni et al., 2004). Diphenyl diselenide, a simple organoselenium compound, has been reported in view of its important pharmacological properties (Nogueira et al., 2003; Nogueira et al., 2004; Borges et al., 2005, 2006; Barbosa et al., 2006; Savegnago et al., 2006, 2007) among them its antioxidant activity (Rossato et al., 2002; Meotti et al., 2004). Recently, studies of our research group have also demonstrated that diphenyl diselenide protects against oxidative damage induced by cadmium in rodents (Santos et al., 2004, 2005a, 2005b).

Based on these reports, this study was designed to determine the possible restorative effect of combined therapy with diphenyl diselenide and DMPS against renal and hematological damage induced by mercury ($HgCl_2$) in mice.

2. Materials and methods

2.1. Materials

Diphenyl diselenide $(PhSe)_2$ was prepared according to the literature method (Paulmier, 1986) and was dissolved in canola oil. Analysis of the ^{1}H NMR and ^{13}C 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. Mercuric chloride ($HgCl_2$), 2,3-dimercaptopropane 1-sulfonate (DMPS), δ-aminolevulinic acid (δ-ALA), 1-chloro-2,4-dinitrobenzene (CDNB) and *p*-dimethylaminobenzaldehyde were purchased from SIGMA (St. Louis, MO, USA). All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.2. Animals

Male adult Swiss albino mice (25-35 g) from our own breeding colony were used. The animals were kept in a separate animal room, on a 12 hour light/dark cycle, at a room temperature of 22 ± 2 ºC and with free access to food and water. This study was approved by the Ethics and Animal Welfare Committee of Universidade Federal de Santa Maria.

2.3. Experimental Procedure

Mice were daily subcutaneously exposed to $HgCl_2$ (1 mg/kg, dissolved in 0.9% NaCl) for two weeks (Booker and White Jr, 2005). After that, mice received by gavage $(PhSe)_2$ (15.6 mg/kg, dissolved in canola oil) (Savegnago et al., 2007) and DMPS (12.6 mg/kg, dissolved in 0.9% NaCl) (Santos et al., 2005b) individually or combined for one week.

The protocol of mouse exposure is given below:

Group 1 - 0.9% NaCl (10 ml/kg, s.c.) followed by canola oil (2.5 ml/kg, p.o.) plus 0.9% NaCl (5 ml/kg, p.o.)

Group 2- 0.9% NaCl (10 ml/kg, s.c.) followed by canola oil (2.5 ml/kg, p.o.) plus DMPS

(5 ml/kg, p.o.)

Group 3- 0.9% NaCl (10 ml/kg, s.c.) followed by $(PhSe)_2$ (2.5 ml/kg, p.o.) plus 0.9%

NaCl (5 ml/kg, p.o.)

Group 4- 0.9% NaCl (10 ml/kg, s.c.) followed by $(PhSe)_2$ (2.5 ml/kg, p.o.) plus DMPS (5

ml/kg, p.o.)

Group 5- $HgCl_2$ (10 ml/kg, s.c.) followed by canola oil (2.5 ml/kg, p.o.) plus 0.9% NaCl (5

ml/kg, p.o.)

Group 6- $HgCl_2$ (10 ml/kg, s.c.) followed by canola oil (2.5 ml/kg, p.o.) plus DMPS (5

ml/kg, p.o.)

Group 7- $HgCl_2$ (10 ml/kg, s.c.) followed by $(PhSe)_2$ (2.5 ml/kg, p.o.) plus 0.9% NaCl (5

ml/kg, p.o.)

Group 8- $HgCl_2$ (10 ml/kg, s.c.) followed by $(PhSe)_2$ (2.5 ml/kg, p.o.) plus DMPS (5

ml/kg, p.o.)

Twenty four hours after the last administration, a sample of heparinized blood sample was taken by cardiac puncture from each anesthetized mouse. After centrifugation (3000xg) for 10 min at 4^oC, the plasma and buffy coat were separated. Subsequently, mice were sacrificed by decapitation and kidneys were removed.

The samples of kidneys were homogenized (1/10, w/v) in 50 mM Tris-HCl (pH 7.4) and centrifuged (2400×g) for 15 min. The sample supernatant (S1) was separated and used for biochemical assays.

2.4. Thiobarbituric acid-reactive substances (TBARS) levels

An aliquot (200 μl) of S1 was incubated at 95°C for 2 h. Renal TBARS levels were determined as described by Ohkawa et al. (1979). The absorbance was read at 532 nm and the data expressed as nmol malondialdehyde (MDA)/g tissue.

2.5. Ascorbic acid levels

Renal ascorbic acid determination was performed as described by Jacques-Silva et al. (2001). The protein content of S1 was precipitated in 10 volumes of a cold 4% trichloroacetic acid solution. An aliquot of sample (300 μl) in a final volume of 1 ml of solution was incubated at 37°C for 3 hours, then 1 ml H_2SO_4 65% (v/v) was added to the medium. The reaction product was determined using color reagent containing 4.5 mg/ml dinitrophenyl hydrazine and $CuSO_4$ (0.075 mg/ml). The absorbance was read at 520 nm and the data expressed as µg ascorbic acid (AA)/g tissue.

2.6. Catalase (CAT) activity

Renal catalase activity was assayed spectrophotometrically by the method of Aebi (1984), which involves monitoring the disappearance of H_2O_2 in the homogenate at 240 nm. Enzymatic reaction was initiated by adding an aliquot of 20 μl of S1 and the substrate (H_2O_2) to a concentration of 0.3 mM in a medium containing 50 mM phosphate buffer, pH 7.0. The enzymatic activity was expressed as UI/mg protein.

2.7. Glutathione S-transferase (GST) activity

Renal GST activity was assayed in S1 through the conjugation of glutathione with CDNB at 340 nm as described by Habig et al. (1974). The results are expressed as µmol/min/mg protein.

2.8. Biochemical parameters in plasma

Plasma lactate dehydrogenase (LDH) activity, estimation of total, direct and indirect billirubin contents as well as urea, creatinine and uric acid levels were assayed spectophotometrically using commercial Kits (LABTEST, Diagnostica S.A., Minas Gerais, Brazil).

2.9. Hematological Parameters

Hematological parameters were evaluated using a Pentra 120 Retic-Count Analyzer™ (ABX Diagnostics, USA). The methods used by this equipment are flow citometry, impedance, photometry and citochemistry.

2.10. Mercury concentration

The samples of kidney were completely digested with 65% nitric acid (Suprapur, Merk). Renal mercury concentration was analyzed by cold vapour atomic absorption spectrometry (CVAAS; model A Analyst 800, Perkin Elmer) coupled to a generator of hydrides (model FIAS Mercury/Hydride, Chemifold). Standard from Fluka was used to

build up the standard curve (1-50 micrograms/l). The results are expressed as µg mercury/g dry weight.

2.11. Statistical analysis

The results are presented as means $\pm$ S.D. Statistical analysis was performed using a three-way ($HgCl_2$ x DMPS x $(PhSe)_2$) analysis of variance (ANOVA), followed by Duncan's multiple range test when appropriate. Values of $p < 0.05$ were considered statistically significant. Main effects or second order interactions are presented only when the higher (third) order interaction was non-significant.

3. Results

3.1. Renal TBARS levels

Three-way ANOVA of TBARS levels data showed a significant $HgCl_2$ x DMPS x $(PhSe)_2$ interaction ($F_{1,39}=19.28$, $p < 0.001$). Post-hoc comparisons demonstrated that DMPS and $(PhSe)_2$, individually or combined, restored TBARS levels increased by $HgCl_2$ (Table 1).

3.2. Ascorbic acid levels

Three-way ANOVA of ascorbic acid levels data did not demonstrate significant alterations (Table 1).

3.3. CAT activity

Three-way ANOVA of CAT activity data did not demonstrate significant alterations (Table 1).

3.4. GST activity

A significant interaction between $HgCl_2$ x DMPS in GST activity data was observed ($F_{1,23}$=5.26, p<0.03). Post-hoc comparisons demonstrated that DMPS restored GST activity stimulated by $HgCl_2$ (Table 1).

3.5. Biochemical parameters in plasma

3.5.1. LDH activity

Three-way ANOVA of plasma LDH activity data yielded a significant $HgCl_2$ x DMPS x $(PhSe)_2$ interaction ($F_{1,40}$=74.50, p<0.001). Post-hoc comparisons demonstrated that DMPS and $(PhSe)_2$ restored LDH activity increased by $HgCl_2$ exposure. Combined treatment with DMPS + $(PhSe)_2$ was capable of restoring LDH activity but not to the levels of the control group (Table 2).

3.5.2. Uric acid concentration

Three-way ANOVA of uric acid levels data showed a significant $HgCl_2$ x DMPS x $(PhSe)_2$ interaction ($F_{1,39}$=14.97, p<0.001). Post-hoc comparisons showed that DMPS and $(PhSe)_2$ restored uric acid levels increased in mice exposed to $HgCl_2$. Combined

therapy with DMPS + $(PhSe)_2$ did not restore uric acid levels increased by $HgCl_2$ (Table 2).

3.5.3. Urea and creatinine levels

Three-way ANOVA of plasma urea levels data yielded a significant $HgCl_2$ x DMPS x $(PhSe)_2$ interaction ($F_{1,40}$=16.84, $p<0.001$). Post-hoc comparisons demonstrated that DMPS and $(PhSe)_2$ restored urea levels increased by $HgCl_2$. Combined therapy with DMPS + $(PhSe)_2$ was not effective in restoring urea levels increased by $HgCl_2$ (Table 2).

Three-way ANOVA of plasma creatinine levels data yielded a significant $HgCl_2$ x DMPS x $(PhSe)_2$ interaction ($F_{1,46}$=125.21, $p<0.001$). Post-hoc comparisons demonstrated that DMPS and $(PhSe)_2$ ameliorated creatinine levels increased by $HgCl_2$ exposure. Combined DMPS + $(PhSe)_2$ treatment restored creatinine levels but not to levels of the control group (Table 2).

3.5.4. Billirubin content

Three-way ANOVA of total billirubin content data yielded a significant DMPS x $(PhSe)_2$ interaction ($F_{1,38}$=4.23, $p<0.046$). DMPS and $(PhSe)_2$ restored total billirubin content increased by $HgCl_2$. Combined treatment with DMPS + $(PhSe)_2$ was not effective in restoring the total billirubin content (Table 3).

Three-way ANOVA of indirect billirubin content data yielded a significant $HgCl_2$ x DMPS x $(PhSe)_2$ interaction ($F_{1,35}$=6.54, $p<0.015$). Post-hoc comparisons demonstrated

that DMPS and (PhSe)$_2$, individually or combined, restored the increase on indirect billirubin content induced by HgCl$_2$ (Table 3).

Three-way ANOVA of direct billirubin content data did not yield significant alterations (Table 3).

3.6. Hematogical Parameters

3.6.1. Erythrocyte, hemoglobin and hematocrit levels

Three-way ANOVA of erythrocyte levels data showed a significant HgCl$_2$ x DMPS x (PhSe)$_2$ interaction ($F_{1,28}$=20.44, p<0.001). Post-hoc comparisons demonstrated that DMPS and (PhSe)$_2$ restored erythrocyte levels reduced by HgCl$_2$. Therapy with DMPS + (PhSe)$_2$ did not restore erythrocyte levels reduced by HgCl$_2$ exposure in mice (Figure 1A).

Three-way ANOVA of hemoglobin levels data yielded a significant HgCl$_2$ x DMPS x (PhSe)$_2$ interaction ($F_{1,27}$=103.21, p<0.001). Post-hoc comparisons demonstrated that DMPS and (PhSe)$_2$ restored hemoglobin levels reduced by HgCl$_2$. Combined treatment with DMPS + (PhSe)$_2$ did not alter HgCl$_2$ effect on hemoglobin levels (Figure 1B).

Three-way ANOVA of hematocrit levels data revealed a significant HgCl$_2$ x DMPS x (PhSe)$_2$ interaction ($F_{1,27}$=66.60, p<0.001). Post-hoc comparisons demonstrated that DMPS and (PhSe)$_2$ restored hematocrit levels reduced by HgCl$_2$. DMPS + (PhSe)$_2$ therapy did not abolish the HgCl$_2$ effect (Figure 1C).

3.6.2. Mean corpuscular volume (VCM), mean corpuscular hemoglobin (HCM), mean corpuscular hemoglobin concentration (MCHC) and red distribution weight (RDW) values.

Three-way ANOVA of VCM, HCM, CHCM and RDW values did not yield significant alterations (Table 4).

3.6.3. Leukocyte and platelet counts

Three-way ANOVA of leukocyte counts data yielded a significant $HgCl_2$ x DMPS x $(PhSe)_2$ interaction ($F_{1,24}$=38.70, p<0.001). Post-hoc comparisons demonstrated that DMPS and $(PhSe)_2$ restored leukocyte counts reduced by $HgCl_2$ exposure. Combined therapy with DMPS + $(PhSe)_2$ did not restore the leukocyte counts reduced by $HgCl_2$ (Figure 2A).

Three-way ANOVA of platelet counts data revealed a significant $HgCl_2$ x DMPS x $(PhSe)_2$ interaction ($F_{1,26}$=70.24, p<0.001). Post-hoc comparisons demonstrated that DMPS and $(PhSe)_2$ restored but not completely platelet counts reduced by $HgCl_2$. Combined therapy was less effective than individual treatments to restore platelet counts reduced by $HgCl_2$ exposure (Figure 2B).

3.6.4. Lymphocyte, monocyte and neuthrophil percentages

Three-way ANOVA of lymphocyte percentage data yielded a significant $HgCl_2$ x DMPS x $(PhSe)_2$ interaction ($F_{1,26}$=107.99, p<0.001). Post-hoc comparisons demonstrated that DMPS and $(PhSe)_2$ completely restored lymphocyte percentage

reduced by $HgCl_2$. In contrast, combined therapy with DMPS + $(PhSe)_2$ potentiated the reduction in lymphocyte percentage induced by $HgCl_2$ (Figure 3A).

Three-way ANOVA of monocyte percentage data revealed a significant $HgCl_2$ x DMPS x $(PhSe)_2$ interaction ($F_{1,19}$=9.18, p<0.007). Post-hoc comparisons demonstrated that DMPS and $(PhSe)_2$, individually or combined, completely restored the monocyte percentage increased by $HgCl_2$ (Figure 3B).

Three-way ANOVA of neuthrophil percentage data yielded a significant $HgCl_2$ x DMPS x $(PhSe)_2$ interaction ($F_{1,20}$=654.33, p<0.001). Post-hoc comparisons demonstrated that DMPS and $(PhSe)_2$ restored neuthrophil percentage increased by $HgCl_2$. Combined therapy with DMPS + $(PhSe)_2$ did not restore neuthophil percentage increased by $HgCl_2$ exposure (Figure 3C).

3.7. Mercury concentration

A significant interaction between $HgCl_2$ x DMPS ($F_{1,16}$=4.78, p<0.044) was observed. Post-hoc comparisons demonstrated that the increase (~23 times) in renal mercury concentration induced by $HgCl_2$ was reduced by DMPS administration. $(PhSe)_2$ did not modify mercury levels (Figure 4).

4. Discussion

Increasing evidence support the hypothesis that simultaneous administration of selenium with a chelating agent leads to reduced effectiveness of the chelators (Juresa et al., 2005). The present study is in agreement with this hypothesis, showing that concomitant administration of DMPS and $(PhSe)_2$ reduced effectiveness of DMPS in

restoring damage induced by $HgCl_2$ in mice. In addition, this study proved that combined therapy with $(PhSe)_2$ and DMPS was less effective than isolated therapies to counteract $HgCl_2$ induced renal and hematological damage.

The kidneys are the primary target organ for accumulation and toxicity of inorganic mercury (Zalups, 1997). Oxidative stress induced by $HgCl_2$ has been previously reported (Lund et al., 1993; Sener et al., 2007). Oxidative stress induced by $HgCl_2$ was evidenced in kidneys of mice by an increase in TBARS levels and the stimulation of GST activity. We can suggests that, as a consequence of lipid peroxidation, biological membranes are affected causing cellular damage, release of LDH to extracellular media and increased activity of this enzyme in the blood (Kaplan and Pesce 1996). Renal damage observed in animals exposed to $HgCl_2$ was also evidenced by an increase in plasma urea, creatinine and uric acid levels, which are renal markers of damage.

It has been found that mercury can cause several hematological alterations, such as hemolysis of red blood cells (Zolla et al., 1997) and alteration in the leukocyte counts (Girardi et al., 1996). In the present study, toxicity induced by $HgCl_2$ was marked by hematological alterations demonstrated by the reduction in erythrocyte, hemoglobin and hematocrit, indicating anemia. To support the hypothesis of hemolytic anemia, billirubin content was evaluated. Mice treated with $HgCl_2$ presented an increase in billirubin content. Billirubin is one of the most sensitive parameters that reflect the prognosis of acute hepatic diseases (Batra and Acharya, 2003). In the present study, the hyperbillirubinemia observed is mostly of the indirect (unconjugated) category. This could be attributable to hemolysis, which is caused by excessively rapid destruction of erythrocytes. This hypothesis was corroborated by the normal values of RDW, which

demonstrated no variance on size of erythrocytes, and VCM, HCM and CHCM values, excluding evidence of iron deficient anemia (Brugnara, 2000).

A reduction in other hematological parameters (platelet, leukocyte and lymphocyte counts) and the increase in neutrofil and monocyte percentages were also found in $HgCl_2$ exposed mice. It is known that mercury can induce abnormal responses in the immune system, including leukocyte count, a marker of cellular defense (Friberg and Enestrom, 1991; WHO, 1991). The increase in neutrofil and monocyte percentages, which represents the activity of the first and second lines of defense against the cellular damage, has been reported after mercury exposure (Contrino et al., 1988; Perlingerio and Queiroz, 1995).

One important finding of this study is that association of $(PhSe)_2$ and DMPS was less effective than individual therapies to restore hematological and renal damage induced by $HgCl_2$. On the one hand, the findings reported in this study support the hypothesis that simultaneous administration of selenium (in the form of $(PhSe)_2$) and a chelating agent (DMPS) leads to reduced effectiveness to remove Hg of the chelator or $(PhSe)_2$. Juresa et al. (2005) have proposed that ligand competition between the chelators and selenoprotein-P led to the redistribution of mercury and decreased urinary excretion.

Given that one can propose that ligand competition between $(PhSe)_2$ and Hg^{2+} to complex with DMPS gives preferentially the complex DMPS-SePh (**1**, Scheme 1). As a consequence of preferential thiol group attack to $(PhSe)_2$ to give two new Se-S bounds, DMPS-Hg^{2+} complex is less probable to occur, reducing Hg^{2+} urinary excretion and the effectiveness of DMPS. In this way, we found that combined therapy with $(PhSe)_2$ and DMPS did not modify the renal mercury levels when compared to DMPS therapy, but

reduced the efficacy of DMPS in restoring hematological and renal damages. One possible explanation for these findings is that in mice exposed to $HgCl_2$ and DMPS the renal mercury may be in the form of DMPS-Hg^{2+} complex ready to be excreted. In contrast, as cited above, combined therapy with $(PhSe)_2$ and DMPS reduces the formation of DMPS-Hg^{2+} complex and, consequently, leave mercury more available to exert toxic effects.

Scheme 1 DMPS - $(PhSe)_2$ complex in the presence of Hg^{+2}
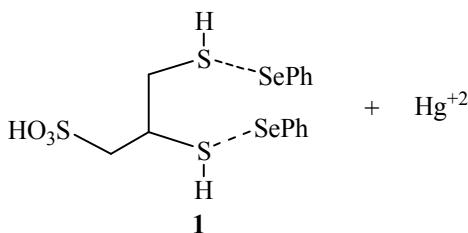
Therefore, this argument helps to explain, at least in part, the reduced effectiveness demonstrated by combined therapy used in this study. Another complicating factor in the relationship between selenium and mercury toxicity, is that GSH is involved in mercury uptake by the kidneys. The increase in GSH levels in different tissues including kidneys has been reported after $(PhSe)_2$ administration in rodents (Barbosa et al., 2006; De Bem et al., 2007; Luchese et al., 2007), so the effect of selenium on GSH levels may also be relevant to the toxic effects of mercury.

The results of this study lead us to refute the hypothesis that administration concomitant of $(PhSe)_2$ and DMPS could ameliorate renal and hematological damage induced by $HgCl_2$ exposure in mice. Although several lines of evidence indicate that

combined therapy with antioxidant and chelating can yield better therapeutic outcomes than isolated chelation therapy (Pande and Flora, 2002; Tandon et al., 2003; Santos et al., 2005b), the findings showed in this study strongly suggested the contrary. Different from our data is also a previous study reporting that administration of DMPS and $(PhSe)_2$ was efficient in ameliorating damage induced by cadmium in mice (Santos et al., 2005b).

The hypotheses proposed to explain the restorative effect of $(PhSe)_2$ against $HgCl_2$ induced damage are: i) the first one is its property of forming an inactive complex with mercury. This hypothesis is supported by studies that demonstrated an inactive complex formation between mercury, selenium (as selenide) and selenoprotein P (Sasakura and Suzuki, 1998; Gailer et al., 2000). In accordance, $(PhSe)_2$ did not modify mercury concentration in kidney of mice exposed to $HgCl_2$ but had restorative effects on hematological and renal alterations induced by $HgCl_2$. Therefore, we suggest that mercury, in the form of an inactive complex, was less available to cause toxic effects in mice exposed to $HgCl_2$ and $(PhSe)_2$. ii) the second one is its antioxidant property (Meotti et al., 2004). In fact, several authors have shown that antioxidants should be one of the important components of an effective treatment against metal poisoning (Casalino et al., 2002; El-Demerdash et al., 2004; Santos et al., 2005b). The restorative effect of DMPS against damage induced by $HgCl_2$ is attributed to chelating activity of this compound which bonds to mercury in a 1:1 ratio, forming a hydrosoluble and less toxic complex (Hg-DMPS), which can be excreted in the urine (Aposhian et al., 1995). In fact, our results confirm the reduction in renal mercury levels in mice exposed to $HgCl_2$ and DMPS.

In conclusion, combined therapy with (PhSe)$_2$ and DMPS was less effective than isolated therapies in restoring renal and hematological damage induced by HgCl$_2$. Therefore, the association of (PhSe)$_2$ and DMPS seems to be not a good choice to counteract the damage induced by HgCl$_2$ in mice. However, more studies are required to clarify this matter.

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Legends

Figure 1. Effect of (PhSe)$_2$ and DMPS on erythrocyte (1A), hemoglobin (1B) and hematocrit (1C) levels from mice exposed to HgCl$_2$. Data are reported as mean ± S.D. of six animals per group. (a) Denoted p<0.05 as compared to the control group (three-way ANOVA/Duncan). (b) Denoted p<0.05 as compared to the HgCl$_2$ group (three-way ANOVA/Duncan). (c) Denoted p<0.05 as compared to the HgCl$_2$ + DMPS and HgCl$_2$ + (PhSe)$_2$ groups (three-way ANOVA/Duncan).

Figure 2. Effect of (PhSe)$_2$ and DMPS on leukocyte (2A) and platelet (2B) levels from mice exposed to HgCl$_2$. Data are reported as mean ± S.D. of six animals per group. (a) Denoted p<0.05 as compared to the control group (three-way ANOVA/Duncan). (b) Denoted p<0.05 as compared to the HgCl$_2$ group (three-way ANOVA/Duncan). (c) Denoted p<0.05 as compared to the HgCl$_2$ + DMPS and HgCl$_2$ + (PhSe)$_2$ groups (three-way ANOVA/Duncan).

Figure 3. Effect of (PhSe)$_2$ and DMPS on lymphocyte (3A), monocyte (3B) and neuthrophil (3C) percentage from mice exposed to HgCl$_2$. Data are reported as mean ± S.D. of six animals per group. (a) Denoted p<0.05 as compared to the control group (three-way ANOVA/Duncan). (b) Denoted p<0.05 as compared to the HgCl$_2$ group (three-way ANOVA/Duncan). (c) Denoted p<0.05 as compared to the HgCl$_2$ + DMPS and HgCl$_2$ + (PhSe)$_2$ groups (three-way ANOVA/Duncan).

Figure 4. Effect of $(PhSe)_2$ and DMPS on renal mercury levels in mice exposed to $HgCl_2$. Data are reported as mean ± S.D. of three animals per group. (a) Denoted $p < 0.05$ as compared to the control group (three-way ANOVA/Duncan). (b) Denoted $p < 0.05$ as compared to the $HgCl_2$ group (three-way ANOVA/Duncan).

Tables

Table 1: Effect of (PhSe)$_2$ and DMPS on renal TBARS and ascorbic acid levels and GST and CAT activities of mice exposed to HgCl$_2$

	TBARS (nmol MDA/mg prot.)	Ascorbic acid (µg AA/g tissue)	CAT (UI/mg prot.)	GST (µmol/min/mg prot.)
Control	50.7 ± 3.4	112.8 ± 14.7	29.9 ± 2.7	2.4 ± 0.7
DMPS	58.1 ± 9.2	122.8 ± 19.4	33.1 ± 5.9	2.5 ± 0.3
(PhSe)$_2$	49.1 ± 5.5	118.1 ± 19.8	37.3 ± 9.0	5.1 ± 1.4 [a]
DMPS+ (PhSe)$_2$	48.7 ± 5.3	130.0 ± 23.3	35.3 ± 8.5	4.2 ± 0.9 [a]
HgCl$_2$	78.6 ± 4.5 [a]	117.9 ± 24.7	34.5 ± 4.0	5.3 ± 1.3 [a]
HgCl$_2$ + DMPS	56.1 ± 3.5 [b]	96.9 ± 8.1	28.0 ± 2.9	3.1 ± 0.6 [b]
HgCl$_2$ + (PhSe)$_2$	51.0 ± 7.9 [b]	120.6 ± 19.3	31.9 ± 3.6	6.6 ± 1.4 [a]
HgCl$_2$ + DMPS + (PhSe)$_2$	51.8 ± 6.4 [b]	130.4 ± 17.7	33.9 ± 5.4	4.6 ± 1.5 [a]

Data are reported as mean ± S.D. of six animals per group.
[a] Denoted $p < 0.05$ as compared to the control group (Three-way ANOVA/Duncan).
[b] Denoted $p < 0.05$ as compared to the HgCl$_2$ group (Three-way ANOVA/Duncan).

Table 2: Effect of (PhSe)$_2$ and DMPS on plasma LDH activity and uric acid, urea and creatinine levels of mice exposed to HgCl$_2$

	LDH (U/L)	Uric acid (mg/dL)	Urea (mg/dL)	Creatinine (mg/dL)
Control	123.8 ± 17.6	3.9 ± 0.4	48.3 ± 6.4	0.11 ± 0.03
DMPS	131.4 ± 19.0	3.6 ± 0.3	42.0 ± 2.0	0.11 ± 0.01
(PhSe)$_2$	140.6 ± 7.4	3.1 ± 0.7	42.7 ± 7.3	0.11± 0.01
DMPS+ (PhSe)$_2$	149.6 ± 26.6	3.3 ± 0.6	43.0 ± 4.7	0.11 ± 0.01
HgCl$_2$	347.8 ± 55.9 [a]	5.7 ± 1.3 [a]	61.2 ± 5.9 [a]	0.34 ± 0.05 [a]
HgCl$_2$ + DMPS	137.2 ± 12.7 [b]	3.5 ± 0.9 [b]	36.2 ± 3.5 [ab]	0.11 ± 0.02 [b]
HgCl$_2$ + (PhSe)$_2$	116.7 ± 14.6 [b]	2.7 ± 0.5 [ab]	49.6 ± 6.9 [b]	0.11 ± 0.01 [b]
HgCl$_2$ + DMPS + (PhSe)$_2$	205.3 ± 44.6 [abc]	4.9 ± 1.5 [ac]	57.2 ± 5.0 [ac]	0.22 ± 0.05 [abc]

Data are reported as mean ± S.D. of six animals per group.
[a] Denoted p<0.05 as compared to the control group (three-way ANOVA/Duncan).
[b] Denoted p<0.05 as compared to the HgCl$_2$ group (three-way ANOVA/Duncan).
[c] Denoted p<0.05 as compared to the HgCl$_2$ + DMPS and HgCl$_2$ + (PhSe)$_2$ groups (three-way ANOVA/Duncan).

Table 3: Effect of $(PhSe)_2$ and DMPS on plasma total, direct and indirect billirubin content of mice exposed to $HgCl_2$

	Total billirubin (mg/dL)	Direct billirubin (mg/dL)	Indirect billirubin (mg/dL)
Control	0.53 ± 0.15	0.22 ± 0.02	0.29 ± 0.10
DMPS	0.53 ± 0.13	0.24 ± 0.08	0.25 ± 0.08
$(PhSe)_2$	0.45 ± 0.07	0.18 ± 0.03	0.27 ± 0.08
DMPS+ $(PhSe)_2$	0.48 ± 0.09	0.24 ± 0.03	0.24 ± 0.04
$HgCl_2$	0.76 ± 0.15 [a]	0.22 ± 0.01	0.57 ± 0.10 [a]
$HgCl_2$ + DMPS	0.59 ± 0.10 [b]	0.22 ± 0.08	0.32 ± 0.10 [b]
$HgCl_2$ + $(PhSe)_2$	0.54 ± 0.17 [b]	0.26 ± 0.02	0.32 ± 0.05 [b]
$HgCl_2$ + DMPS + $(PhSe)_2$	0.67 ± 0.16	0.28 ± 0.04	0.36 ± 0.07 [b]

Data are reported as mean ± S.D. of six animals per group.
[a] Denoted $p<0.05$ as compared to the control group (three-way ANOVA/Duncan).
[b] Denoted $p<0.05$ as compared to the $HgCl_2$ group (three-way ANOVA/Duncan).

Table 4: Effect of (PhSe)$_2$ and DMPS on VCM, HCM, CHCM and RDW values of mice exposed to HgCl$_2$

	VCM (μm^3)	HCM (pg)	CHCM (g/dL)	RDW (%)
Control	60.8 ± 6.9	28.7 ± 0.9	31.5 ± 1.5	14.0 ± 0.1
DMPS	59.7 ± 9.2	28.7 ± 1.1	32.1 ± 0.8	14.0 ± 0.1
(PhSe)$_2$	59.8 ± 9.2	28.7 ± 0.5	31.3 ± 1.5	14.2 ± 0.9
DMPS+ (PhSe)$_2$	59.8 ± 7.2	28.2 ± 0.9	31.8 ± 1.1	14.2 ± 0.9
HgCl$_2$	61.6 ± 7.7	28.5 ± 0.6	30.4 ± 2.3	15.0 ± 1.4
HgCl$_2$ + DMPS	60.5 ± 9.7	29.0 ± 0.1	31.5 ± 1.4	14.3 ± 0.5
HgCl$_2$ + (PhSe)$_2$	60.4 ± 7.2	28.5 ± 1.3	31.4 ± 0.6	14.2 ± 0.5
HgCl$_2$ + DMPS + (PhSe)$_2$	60.5 ± 10.4	29.0 ± 1.0	31.7 ± 1.6	14.3 ± 0.5

Data are reported as mean ± S.D. of six animals per group.

Figures

Figure 1

A

B

C
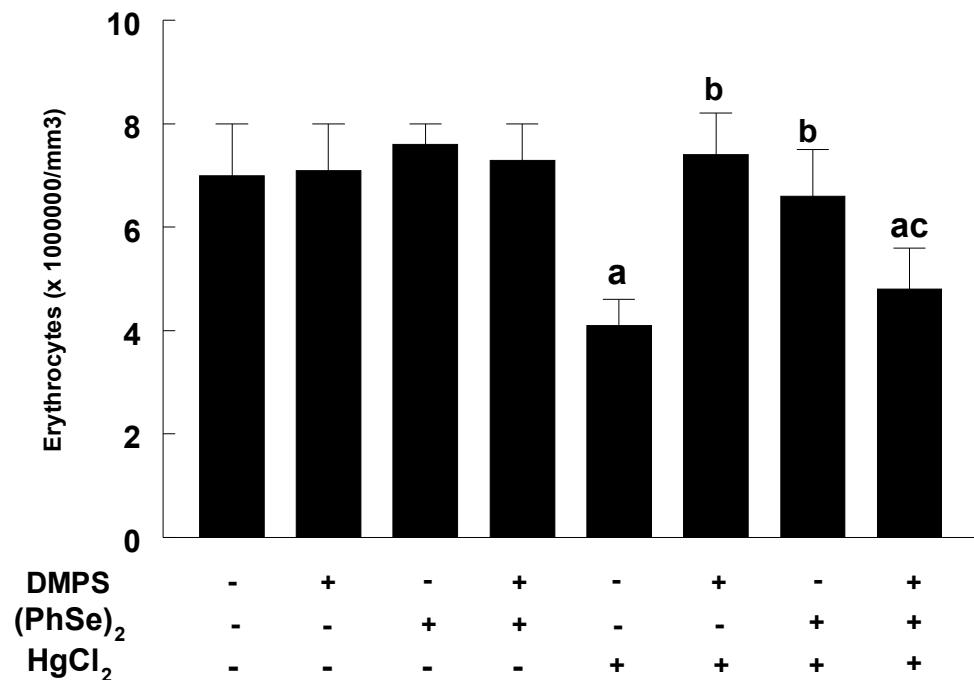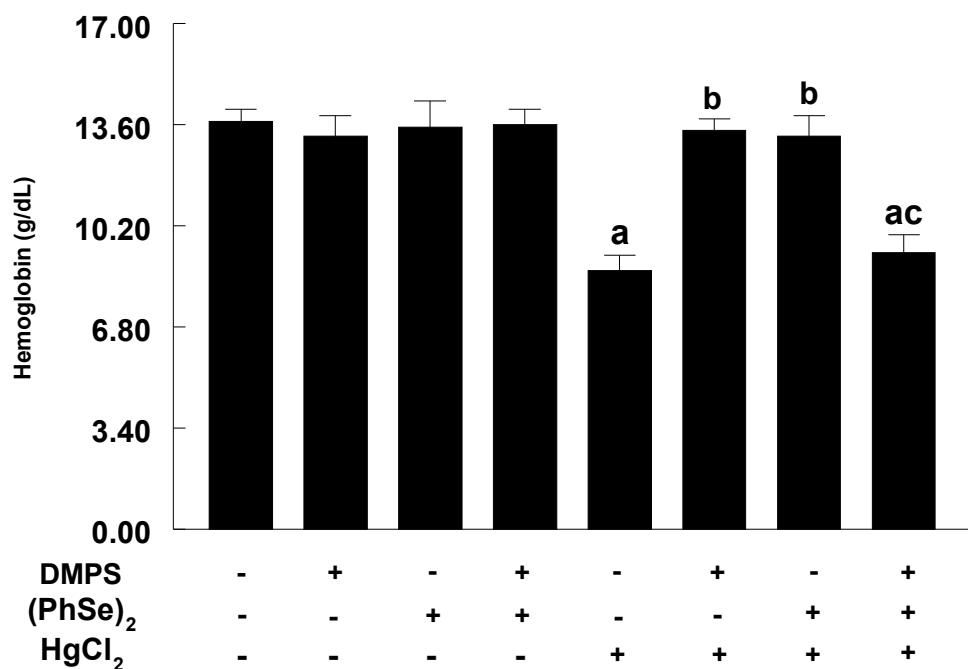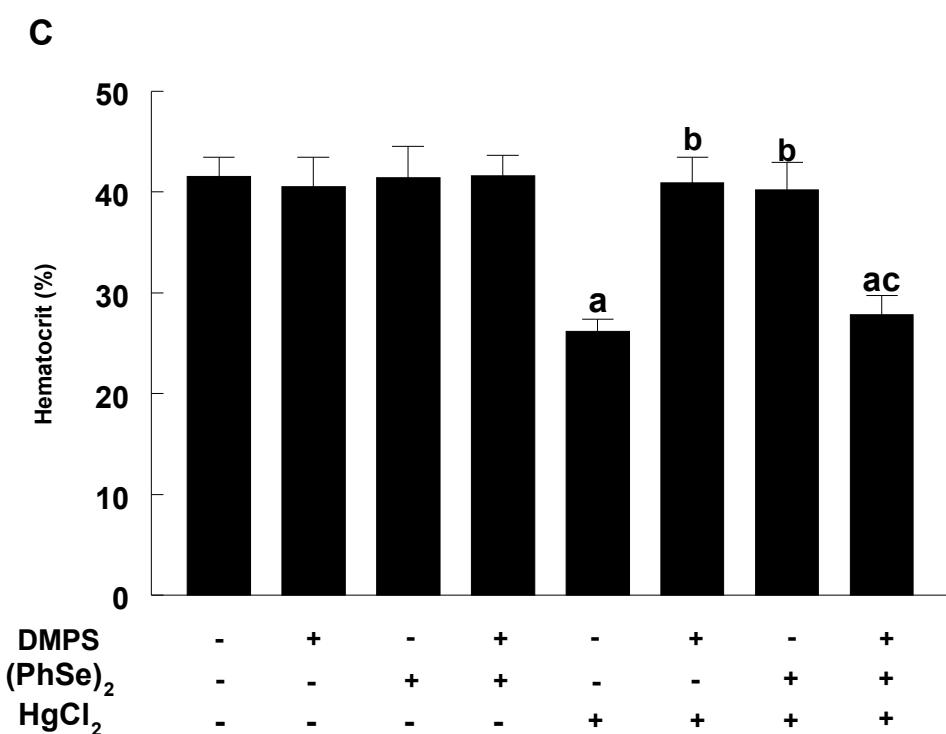
Figure 2

Figure 3

A

B

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Figure 4

|								
DMPS	-	-	+	+	-	-	+	+
(PhSe)$_2$	-	+	-	+	-	+	-	+
HgCl$_2$	-	-	-	-	+	+	+	+
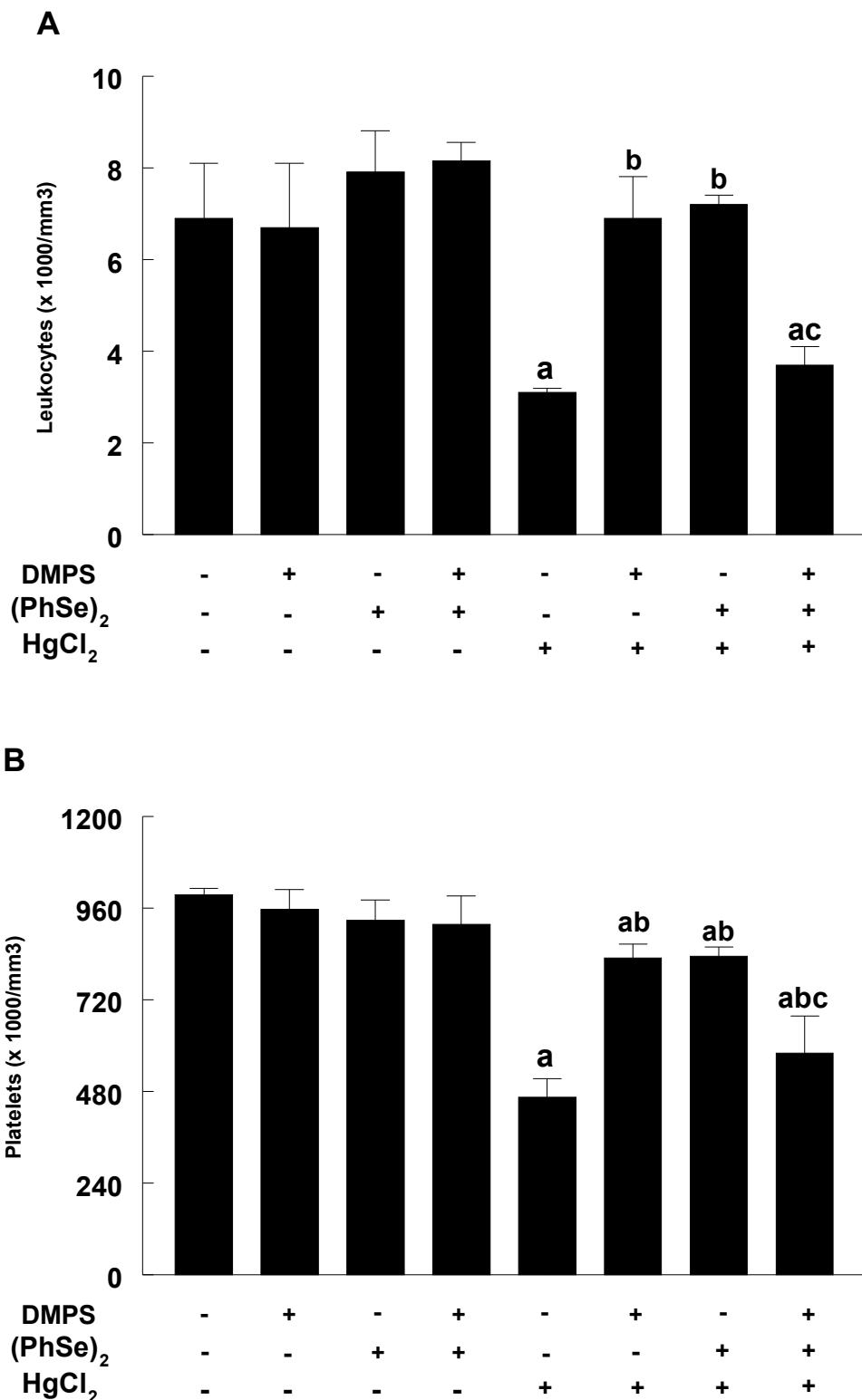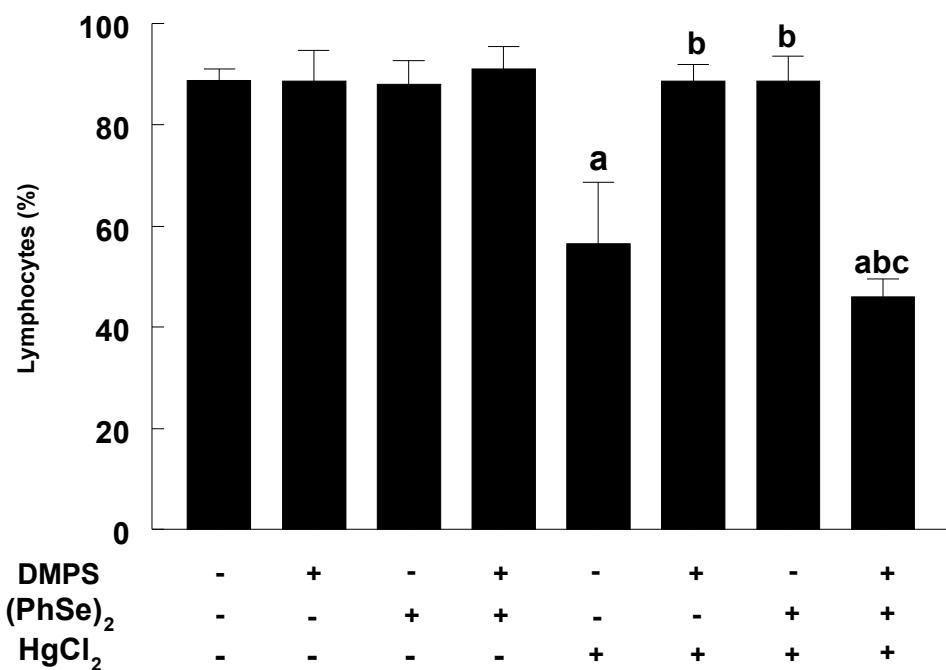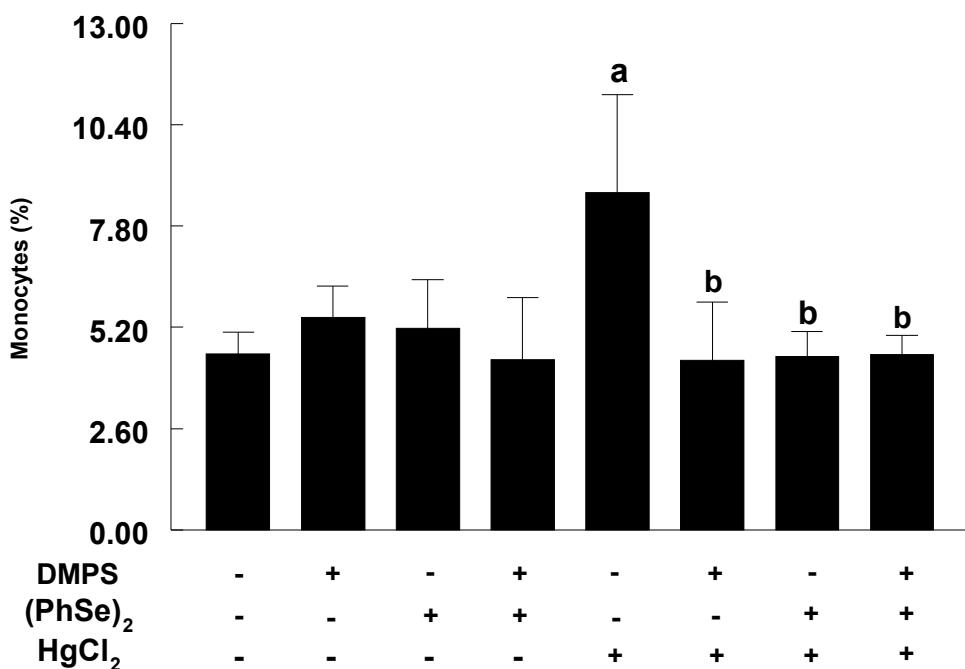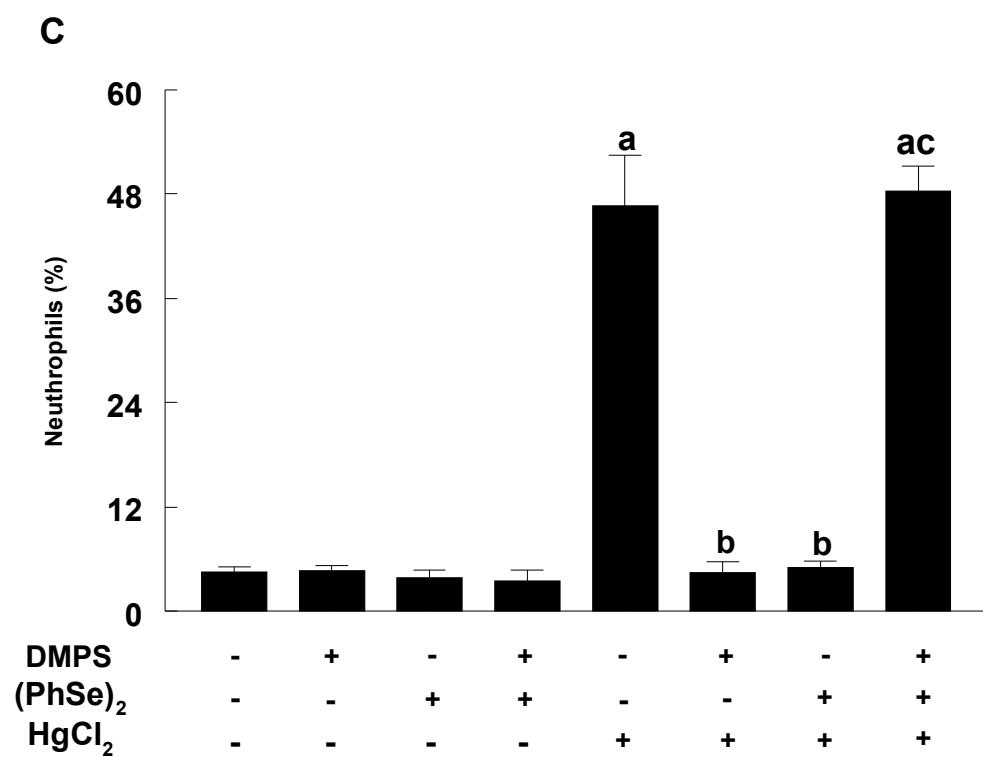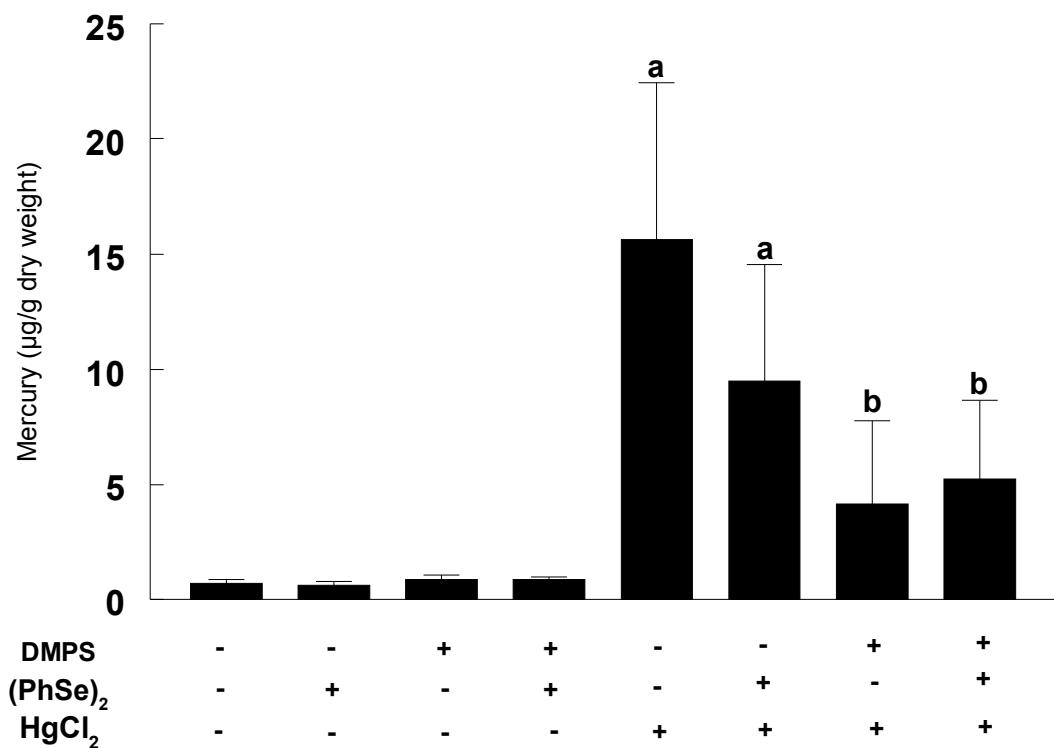
## 5. DISCUSSÃO

Muitos estudos têm sido realizados com o objetivo de elucidar os efeitos da interação entre o mercúrio e o selênio no organismo de mamíferos. Tem sido documentado que a administração de compostos de selênio pode tanto reduzir a toxicidade do mercúrio (Magos e Webb, 1980; Cuvin-Aralar and Furness, 1991; El-Demerdash, 2001; Farina et al., 2003) quanto apresentar um efeito sinérgico ou aditivo com este metal (Magos, 1991; Farina et al., 2004; Brandão et al., 2005). Neste estudo, observamos efeitos distintos da administração de $(PhSe)_2$ em camundongos expostos ao $HgCl_2$. É importante ressaltar que a diferença entre os protocolos experimentais pode ser uma explicação para os resultados distintos observados nestes trabalhos.

Os efeitos tóxicos do mercúrio são bastante relatados na literatura e o estresse oxidativo parece estar envolvido nesta toxicidade (Hussain et al., 1999; Mahboob et al., 2001). Desta forma, no **Artigo** 1, verificamos o efeito de dois compostos com ação antioxidante, o $(PhSe)_2$ e a NAC, em comparação com um agente quelante de referência, o DMPS, na toxicidade aguda induzida pelo $HgCl_2$ em camundongos. Os resultados obtidos demonstraram claramente que a interação dos agentes terapêuticos testados com o mercúrio causou efeitos tóxicos nos camundongos. Observamos, 24 horas após o término do tratamento, uma taxa de 100% de letalidade nos animais do grupo $HgCl_2$ + $(PhSe)_2$, sugerindo um efeito sinérgico entre estes dois compostos. Os efeitos tóxicos observados em animais expostos ao mercúrio e ao selênio têm sido atribuidos, em parte, aos efeitos pró-oxidantes desta interação (Brandão et al., 2005). A formação de um complexo entre mercúrio e selênio pode ser a responsável por esta toxicidade. De fato, Moller-Madsen e Danscher (1991) têm indicado que pode haver a formação de complexos insolúveis denominados de seleneto de mercúrio (HgSe). Além disso, Santos et al. (2005) demonstraram que o $(PhSe)_2$ pode se complexar com o cádmio.

Com relação a NAC e ao DMPS, verificamos a falta de efeitos terapêuticos destes dois agentes e, além disso, demonstramos a indução de toxicidade renal nos animais expostos ao $HgCl_2$ e tratados com NAC ou DMPS. A toxicidade renal observada nos camundongos destes grupos foi demonstrada através de marcadores de

dano renal (uréia e creatinina) e através da atividade da enzima Na^+, K^+-ATPase renal. Sabe-se que esta é uma enzima sensível a agentes oxidantes, uma vez que possui grupos –SH em sua estrutura (Anner et al., 1990). Como descrito anteriormente, o DMPS é um agente quelante e, portanto, pode se complexar com o mercúrio (Zalups et al., 1998). Além disso, a NAC também pode possuir ação quelante (Banner et al., 1986). É descrito na literatura que complexos formados entre o mercúrio e as moléculas tiólicas, como a NAC e o DMPS, podem apresentar propriedades pró-oxidantes (Miller e Woods, 1993; Putzer et al., 1995). Desta forma, sugerimos que a atividade da enzima Na^+, K^+-ATPase renal tenha sido inibida pela ação pró-oxidante dos complexos Hg-NAC e Hg-DMPS, os quais poderiam oxidar os grupos –SH da enzima. Uma outra explicação para a maior toxicidade observada nos animais tratados com $HgCl_2$ + NAC e $HgCl_2$ + DMPS é o fato de que os complexos formados entre o mercúrio e as moléculas tiólicas são mais facilmente transportados para o interior do tecido renal (Zalups e Barfuss, 1995a; b). Este transporte é fundamental para a excreção do metal na urina, mas Zalups e Barfuss (1996) demonstraram que o aumento na concentração de mercúrio no tecido renal pode causar um acúmulo, e não excreção, deste metal pelos rins, aumentando a toxicidade do mercúrio.

Como observamos no **Artigo 1**, 100% dos animais tratados com $HgCl_2$ + $(PhSe)_2$ morreram. Com o objetivo de verificar os efeitos tóxicos causados por este tratamento, realizamos um estudo (**Manuscrito 1**) utilizando o mesmo protocolo de exposição referido no **Artigo 1**. Uma vez que os animais não sobreviviam mais do que 5 horas após o término do tratamento, escolhemos o tempo de 5 horas para o sacrifício dos animais. Observamos, no **Manuscrito 1**, que os efeitos tóxicos decorrentes da interação entre o mercúrio e o selênio afetam o tecido hepático e, principalmente, o renal. Os efeitos tóxicos observados no tecido hepático, decorrentes desta interação, foram evidenciados através do aumento nos níveis de TBARS, um marcador de peroxidação lipídica (Huang et al., 1996), e da inibição da atividade da catalase, uma enzima antioxidante (Benov et al., 1990). Dessa forma, sugere-se que o estresse oxidativo esteja envolvido na toxicidade hepática induzida pela interação formada entre o mercúrio e o selênio.

No **Manuscrito 1**, observamos um número de parâmetros renais alterados no grupo $HgCl_2$ + $(PhSe)_2$. Da mesma forma que no fígado, a indução do dano renal parece estar relacionada com o estresse oxidativo. As enzimas sulfidrílicas δ-ALA-D e Na^+, K^+- ATPase, as quais são sensíveis a agentes pró-oxidantes (Anner et al., 1990; Emanuelli et al., 1996), foram inibidas nos animais expostos ao $HgCl_2$ e tratados com $(PhSe)_2$. Este mesmo grupo de animais apresentou alterações nos marcadores de estresse oxidativo (aumento nos níveis de TBARS e ácido ascórbico e inibição da GST). A confirmação do dano renal veio através de marcadores plasmáticos (uréia e proteína) e urinários (proteína e glicose), comumente utilizados na clínica. O dano renal observado neste estudo também levou ao aumento nos níveis de hemoglobina e no hematócrito, já que as proteínas plasmáticas, responsáveis pela regulação da volemia (Houpt e Yang, 1995), encontravam-se diminuídas. Como citado anteriormente, a toxicidade observada nos casos de interação entre o selênio e o mercúrio está bastante relacionada com a formação do complexo Hg-Se, o qual pode apresentar efeitos pró-oxidantes. Além disso, sabe-se que ambos os compostos (mercúrio e selênio) podem apresentar efeitos pró-oxidantes, quando administrados individualmente (Huang et al., 1996; Nogueira et al., 2003c). Dessa forma, um efeito sinérgico ou aditivo não pode ser descartado. Uma outra hipótese que poderia explicar a toxicidade decorrente desta interação é que o $(PhSe)_2$ pode causar um aumento nos níveis de GSH (Barbosa et al., 2006; Luchese et al., 2007). De fato, os resultados mostrados no **Manuscrito 1** indicam um aumento nos níveis de tióis não protéicos (NPSH) em fígado e rim de camundongos expostos ao $(PhSe)_2$ (dados não mostrados). Moléculas tiólicas, como a GSH, podem facilitar a entrada do mercúrio no tecido renal e, desta forma, poderiam aumentar a toxicidade do metal neste tecido (Zalups e Barfuss, 1995a). De fato, Agarwal e Behari (2007) observaram que o selênio, na forma de selenito de sódio, aumenta os níveis de mercúrio em fígado e rim de ratos. No entanto, esta hipótese parece ser menos provável, já que a concentração de mercúrio no fígado e rim de animais expostos ao mercúrio não foi alterada pela administração de $(PhSe)_2$.

Com base nos resultados obtidos no **Artigo 1** e no **Manuscrito 1**, verificamos a toxicidade da interação entre o mercúrio e moléculas que possuem a capacidade de se complexar com este metal. O potencial pró-oxidante destes complexos parece estar

envolvido nos danos referidos acima. É importante ressaltar que, nestes estudos, as administrações de $HgCl_2$ e dos agentes testados (NAC, DMPS e $(PhSe)_2$) foram de forma aguda e a administração destes agentes foi concomitante com a última dose de $HgCl_2$. Uma vez que a exposição aguda, bem como de forma concomitante, parece propiciar a formação de complexos pró-oxidantes, o nosso próximo objetivo foi verificar o efeito do pré e pós-tratamento com $(PhSe)_2$ em camundongos expostos subcronicamente ao $HgCl_2$ (**Artigo 2 e Manuscrito 2**, respectivamente). No **Artigo 2**, verificamos o efeito do pré-tratamento com o $(PhSe)_2$ na toxicidade induzida pelo $HgCl_2$ nos sistemas imunológico e hematológico de camundongos. Já no **Manuscrito 2**, verificamos o efeito da terapia com o $(PhSe)_2$ na tentativa de reverter os danos no tecido renal e no sistema hematológico causados pelo $HgCl_2$ em camundongos. Como a terapia de reversão da toxicidade de metais pesados envolve a utilização de agentes quelantes, no **Manuscrito 2** também comparamos o efeito do $(PhSe)_2$ com um agente quelante de referência, o DMPS. Além disso, verificamos o efeito da interação entre o $(PhSe)_2$ e o DMPS nestes animais.

No **Artigo 2** e no **Manuscrito 2**, verificamos a presença de anemia nos camundongos tratados com o $HgCl_2$, sendo provavelmente devida aos efeitos pró-oxidantes do metal. O mercúrio é descrito por causar inibição de enzimas antioxidantes eritrocitárias importantes (Ribarov e Benov, 1982), reduzir os níveis de GSH eritrocitária (Ribarov e Benov, 1981) e pode ainda gerar EROs através do complexo mercúrio-hemoglobina (Zolla et al., 1997). Estes efeitos do mercúrio podem levar a processos hemolíticos, causando a anemia. De fato, no **Manuscrito 2**, a hipótese de anemia hemolítica foi fortalecida devido ao aumento nos níveis séricos de bilirrubina indireta (ou não-conjugada) nos camundongos expostos ao $HgCl_2$, uma vez que, no processo hemolítico, há liberação do heme que é convertido em bilirrubina indireta.

Outros parâmetros hematológicos (plaquetas e leucócitos) também foram alterados nos camundongos expostos ao $HgCl_2$. As alterações observadas nos leucócitos também refletem o efeito deste metal no sistema imunológico, já que os leucócitos são células de defesa. De fato, observamos que o mercúrio causou alterações nos níveis de dois anticorpos, a imunoglobulina G (IgG) e imunoglobulina M (IgM) (**Artigo 2**). Este efeito do mercúrio é descrito como um dos responsáveis pela

nefropatia observada em animais expostos a este metal. Estudos demonstraram que o mercúrio pode causar doença auto-imune, ou seja, o sistema imunológico produz anticorpos contra suas próprias proteínas e estes se depositam nos rins podendo causar desordens neste tecido (Zelikoff e Thomas, 1998). De acordo com estes relatos, confirmamos no **Manuscrito 2** a presença de dano renal após tratamento subcrônico com o $HgCl_2$ em camundongos. A presença de dano renal foi confirmada através dos marcadores plasmáticos (uréia, creatinina e ácido úrico) e parece estar relacionada com o estresse oxidativo. Diversos trabalhos têm demonstrado que, em humanos e outros mamíferos, os rins são os alvos primários onde os íons mercúricos se acumulam após a exposição às formas elementar e inorgânica deste metal (Cherian e Clarkson, 1976; Zalups e Diamond, 1987; Zalups e Barfuss, 1990).

Os resultados obtidos nestes dois últimos trabalhos (**Artigo 2** e **Manuscrito 2**) foram diferentes dos observados nos dois primeiros trabalhos (**Artigo 1** e **Manuscrito 1**). O $(PhSe)_2$ foi efetivo tanto em proteger quanto em reverter os danos observados nos animais expostos ao $HgCl_2$. Como os danos causados pelo mercúrio envolvem a geração de estresse oxidativo, sugerimos que o efeito benéfico deste organocalcogênio foi devido a sua ação antioxidante, a qual é bastante relatada (Meotti et al., 2004; Nogueira et al., 2004; Luchese et al., 2007; Borges et al., 2008). Sabe-se que o selênio é componente de algumas enzimas que apresentam ação antioxidante, como a glutationa peroxidase (Wingler e Brigelius-Flohé, 1999) e a tiorredoxina redutase (Holmgren, 1985). Além disso, tem sido demonstrado que o $(PhSe)_2$ pode possuir ação semelhante a da glutationa peroxidase (Nogueira et al., 2004) e que este composto pode causar aumento nos níveis de GSH, a qual possui ação antioxidante. Também tem sido documentada a presença de um complexo formado entre mercúrio, selênio e selenoproteína P, o qual seria inerte e, desta forma, reduziria a toxicidade do metal (Yoneda e Suzuki, 1997).

É importante salientar que, no **Manuscrito 2**, observamos que o $(PhSe)_2$ foi praticamente tão efetivo quanto o DMPS em reverter os danos causados pelo mercúrio. Entretanto, a associação do $(PhSe)_2$ ao DMPS reduziu a efetividade deste agente quelante. Embora existam estudos que descrevam a eficácia da associação entre agentes antioxidantes e quelantes (Pande e Flora, 2002; Tandon et al., 2003), nosso

resultado está de acordo com evidências que indicam que a administração de outras formas de selênio reduz a efetividade do DMPS (Juresa et al., 2005). Sugerimos que o (PhSe)$_2$ poderia reduzir a efetividade do DMPS em se complexar com o mercúrio, uma vez que a formação de um complexo entre o (PhSe)$_2$ e o DMPS (DMPS-SePh) poderia ocorrer mais facilmente (Esquema 1 do **Manuscrito 2**). Embora não tenhamos observado alteração na concentração de mercúrio nos animais que receberam a terapia combinada (DMPS + (PhSe)$_2$) em comparação com os animais tratados com o DMPS, nos animais tratados com a associação entre os dois compostos o mercúrio, provavelmente, estaria mais disponível para exercer seus efeitos tóxicos.

A análise destes 4 trabalhos aqui mencionados nos permite entender um pouco mais sobre a interação entre o selênio e o mercúrio. Os resultados sugerem que a administração concomitante de selênio, na forma de (PhSe)$_2$ e mercúrio, na forma de HgCl$_2$, pode causar inúmeros efeitos tóxicos. Entretanto, quando o organocalcogênio é administrado antes ou depois da exposição ao HgCl$_2$, os resultados parecem ser mais efetivos. Uma explicação para os diferentes resultados obtidos pode ser a formação do complexo pró-oxidante, o que talvez seja facilitado pela administração concomitante de HgCl$_2$ e (PhSe)$_2$. Além disso, a exposição subcrônica ao HgCl$_2$ parece responder melhor a terapia com o (PhSe)$_2$ do que a exposição aguda em camundongos. Estes resultados são corroborados por prévios estudos do nosso grupo que demonstraram que a administração de (PhSe)$_2$, mesmo não sendo concomitante com o HgCl$_2$, apresentou poucos efeitos terapêuticos em animais tratados de forma aguda com HgCl$_2$ (Brandão et al., 2006). É importante salientar que nos dois primeiros trabalhos, os quais demonstraram efeitos tóxicos da interação entre (PhSe)$_2$ e HgCl$_2$, o organocalcogênio foi administrado pela via subcutânea. Já nos outros dois trabalhos, os quais demonstraram efeitos benéficos da interação entre (PhSe)$_2$ e HgCl$_2$, o composto de selênio foi administrado pela via oral, o que poderia sugerir que a via de administração fosse fator determinante para a toxicidade ou não desta associação entre o (PhSe)$_2$ e o HgCl$_2$. Entretanto, não podemos afirmar que a via oral é melhor do que a via subcutânea, já que observamos que, utilizando-se o mesmo protocolo de exposição dos dois primeiros trabalhos, a terapia oral com (PhSe)$_2$, na mesma dose que foi usada

subcutaneamente, também causou 100% de morte nos camundongos expostos ao HgCl$_2$ (dados não publicados).

No **Esquema 1**, mostrado a seguir, é possível ter uma visão geral dos efeitos do mercúrio, do (PhSe)$_2$ e dos demais agentes testados (NAC e DMPS), bem como suas interações estudadas neste trabalho.

Esquema 1. Visão geral dos efeitos do mercúrio, do (PhSe)$_2$ e dos demais agentes testados (NAC e DMPS), bem como suas interações estudadas neste trabalho.
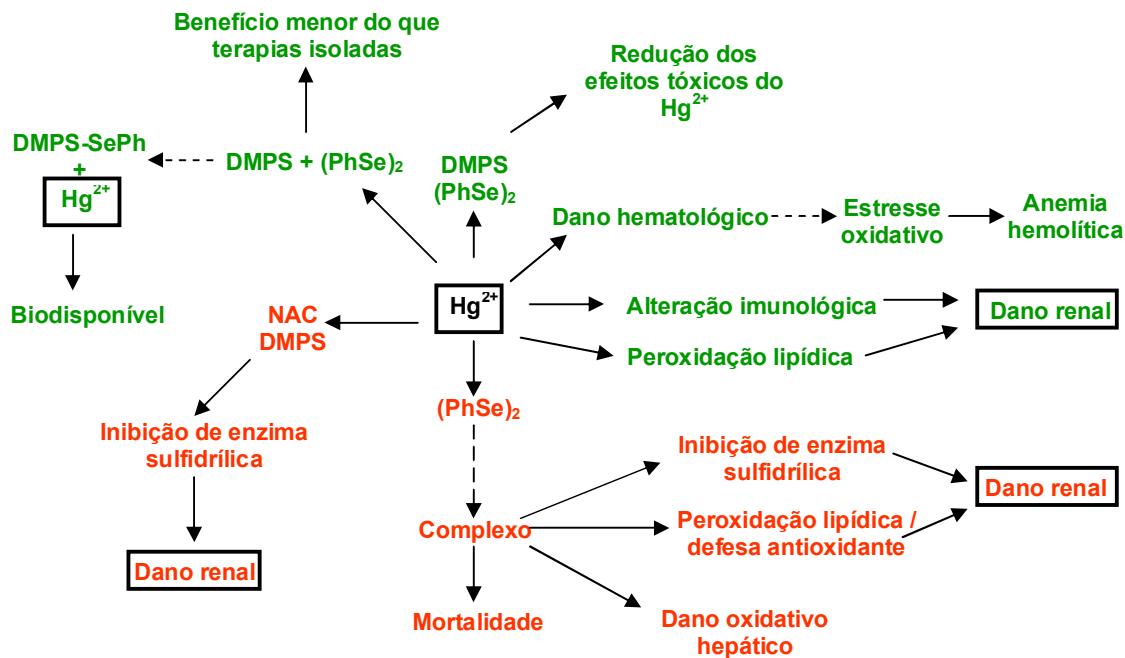
- As linhas cheias (—) indicam os efeitos demonstrados neste trabalho.

- As linhas pontilhadas (---) indicam possíveis efeitos, baseado em estudos da literatura.

- Cor vermelha: tratamento agudo / concomitante.

- Cor verde: tratamento subcrônico / pré e pós.

6. CONCLUSÕES

Com base nos resultados obtidos, podemos concluir que:

• **O Artigo 1** demonstrou que a administração de $(PhSe)_2$, NAC e DMPS, concomitantemente a exposição ao $HgCl_2$, causou toxicidade nos animais expostos de forma aguda ao $HgCl_2$. O $(PhSe)_2$ causou 100% de morte nos camundongos expostos ao metal e a administração de NAC e DMPS apresentou toxicidade renal nos camundongos. Esta toxicidade pode ser mediada através da formação de complexos pró-oxidantes entre o mercúrio e estes agentes e afetam, em especial, o tecido renal;

• O **Manuscrito 1** demonstrou que a toxicidade observada nos camundongos expostos ao $HgCl_2$ e ao $(PhSe)_2$ envolve a geração de estresse oxidativo. A formação de um complexo entre o $(PhSe)_2$ e o $HgCl_2$ é, provavelmente, a causa desta toxicidade que afeta o tecido hepático e, principalmente, o tecido renal;

• O **Artigo 2** demonstrou que o $(PhSe)_2$ possui a capacidade de proteger contra os danos aos sistemas imunológico e hematológico induzidos pela exposição subcrônica ao $HgCl_2$. Este mecanismo de proteção pode incluir a formação de um complexo ternário inerte e também pode ser devido à ação antioxidante deste composto de selênio;

• O **Manuscrito 2** demonstrou que o $(PhSe)_2$ possui efeitos semelhantes aos do DMPS na tentativa de reverter os danos aos sistemas hematológico e renal induzidos pela exposição subcrônica ao $HgCl_2$. O mecanismo pelo qual o $(PhSe)_2$ reverte a toxicidade induzida pelo $HgCl_2$ pode incluir a formação de um complexo ternário inerte e também pode ser devido à ação antioxidante deste composto de selênio. Entretanto, a administração conjunta de $(PhSe)_2$ e DMPS não parece uma boa opção, uma vez que as administrações individuais destes dois compostos foram mais eficazes do que a administração combinada em reverter a toxicidade induzida pelo metal.

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