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ESTUDO DOS QUELANTES SULFIDRÍLICOS (BAL, DMPS E DMSA) E DO DISSELENETO DE DIFENILA: EFEITOS SOBRE PARÂMETROS TOXICOLÓGICOS E MODELOS DE INTOXICAÇÃO POR CÁDMIO

Francielli Weber Santos

Santa Maria, RS, Brasil

2005

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por

Francielli Weber Santos

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

Santa Maria, RS, Brasil

2005

Universidade Federal de Santa Maria

Centro de Ciências Naturais e Exatas

Programa de Pós-Graduação em Bioquímica Toxicológica

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Elaborada por **Francielli Weber Santos** 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

ESTUDO DOS QUELANTES SULFIDRÍLICOS (BAL, DMPS E DMSA): EFEITOS SOBRE PARÂMETROS TOXICOLÓGICOS E MODELOS DE INTOXICAÇÃO POR CÁDMIO

AUTORA: Francielli Weber Santos
ORIENTADORA: Cristina Wayne Nogueira
DATA E LOCAL DA DEFESA: Santa Maria, janeiro de 2005.

Diversos metais alteram as funções celulares por serem capazes de se ligar a grupos tióis de biomoléculas. Portanto, uma terapia eficaz na intoxicação por metal seria a remoção dos metais tóxicos dos bioligantes funcionais através da administração de quelantes contendo grupos tióis. Entretanto, estes compostos poderiam remover metais endógenos, os quais são componentes essenciais de muitos sistemas enzimáticos. Dessa forma, o presente estudo foi delineado para avaliar os efeitos dos agentes quelantes, ácido meso-2,3-dimercaptosuccínico (DMSA), ácido 2,3-dimercaptopropano 1-sulfônico (DMPS) and 2,3-dimercaptopropanol (BAL) *per se* sobre alguns parâmetros toxicológicos e em modelos de intoxicação por cádmio. A atividade da δ-aminolevulinato desidratase (δ-ALA-D) de sangue humano foi inibida pelos agentes quelantes ditiólicos de maneira dependente da concentração. O ditiotreitol e o cloreto de zinco foram efetivos em proteger da inibição da δ-ALA-D causada pelo BAL, DMPS e DMSA de maneira dependente da concentração. O tratamento agudo com os agentes quelantes causou alterações em vários parâmetros toxicológicos em camundongos. O BAL causou uma diminuição significante na atividade da δ-ALA-D renal e um aumento na atividade da enzima cerebral e hepática. O DMPS causou uma inibição na atividade da δ-ALA-D de rim, enquanto os animais que receberam uma única dose de DMSA não apresentaram modificação na atividade da enzima nos tecidos. Todos os quelantes produziram um aumento na peroxidação lipídica no fígado e no rim. Os camundongos que receberam o DMPS apresentaram um aumento na concentração de zinco renal e uma depleção do zinco hepático ocorreu nos camundongos administrados com BAL. De todos os metais tóxicos encontrados no ambiente e utilizados industrialmente, o cádmio ocupa um lugar especial devido à natureza geralmente intratável da intoxicação por este metal. Muitas evidências indicam que as espécies reativas de oxigênio estão envolvidas no dano tecidual induzido pelo cádmio. Então, acredita-se que um antioxidante seja um componente importante de um tratamento efetivo da intoxicação por cádmio, bem como a terapia combinada com antioxidantes e quelantes poderia ser melhor do que a terapia que utiliza apenas os quelantes. A intoxicação aguda por cádmio causou inibição das atividades da δ-ALA-D e da superóxido dismutase (SOD), redução na concentração de ácido ascórbico e aumento da peroxidação lipídica nos testículos dos camundongos. Observou-se também uma elevação da LDH, AST e ALT plasmática. O DMSA, o DMPS e o disseleneto de difenila ($(PhSe)_2$) protegeram do efeito inibitório induzido pelo cádmio na δ-ALA-D e restauraram o aumento dos níveis de espécies reativas ao ácido tiobarbitúrico (TBARS). Entretanto, estes compostos sozinhos ou em combinação

foram incapazes de proteger a atividade da SOD e em recuperar os níveis de ácido ascórbico. A terapia combinada (DMSA e $(PhSe)_2$) não foi melhor que a monoterapia em restaurar os parâmetros toxicológicos avaliados neste modelo de dano testicular induzido por cádmio. Por outro lado, o uso da terapia combinada (DMPS e $(PhSe)_2$) foi melhor que a monoterapia em reduzir os níveis de cádmio nos testículos e em restaurar o nível da AST plasmática. A intoxicação sub-crônica por cádmio em camundongos causou inibição da atividade da δ-ALA-D do fígado, rim e baço e a terapia com $(PhSe)_2$ foi efetiva em restaurar a atividade da enzima em todos os tecidos. Uma redução no conteúdo de ácido ascórbico no rim e no baço foi também observado, enquanto o $(PhSe)_2$ foi efetivo em restaurar esta redução somente no rim. Além disso, a terapia com este composto foi efetiva em restaurar o aumento dos níveis de TBARS causado pelo cádmio nos tecidos hepático e cerebral. De maneira geral, os resultados deste estudo indicam que é necessário investigar a toxicidade potencial dos agentes quelantes, uma vez que eles podem ser tão prejudiciais quanto os metais que eles quelam. Além disso, a terapia com $(PhSe)_2$ na intoxicação por cádmio pode ser útil, tanto quando utilizada sozinha (antioxidante) quanto combinado com DMPS ou DMSA.

Palavras-chave: BAL, DMSA, DMPS, eritrócito, ALA-D, cádmio, disseleneto de difenila, camundongos.

ABSTRACT

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

STUDY OF SULFHYDRYL CHELATORS (BAL, DMPS AND DMSA): EFFECTS ON TOXICOLOGICAL PARAMETERS AND MODELS OF CADMIUM INTOXICATION

AUTHOR: Francielli Weber Santos
ADVISOR: Cristina Wayne Nogueira
DATE AND PLACE OF THE DEFENSE: Santa Maria, 2005

Several metals are known to disturb cellular functions by binding to thiol groups of biomolecules. Therefore, a possible therapy for metal intoxication is to remove the toxic metals from the bound functional bioligands by administering strong thiol-containing chelators. However, these compounds could remove endogenous metals, which are essential components of many enzyme systems. Thus, the present study was designed to evaluate the effects of chelating agents, meso-2,3-dimercaptosuccinic acid (DMSA), 2,3-dimercaptopropane 1-sulfonate (DMPS) and 2,3-dimercaptopropanol (BAL) *per se* on toxicological parameters and models of cadmium intoxication. δ-Aminolevulinate dehydratase (δ-ALA-D) activity from human erythrocytes was inhibited by dithiol chelating agents in a concentration-dependent manner. Dithiotreitol and zinc chloride were able to protect the δ-ALA-D inhibition caused by BAL, DMPS and DMSA in a concentration-dependent manner. Acute treatment with chelating agents caused changes in a number of toxicological parameters in mice. BAL caused a decrease on renal δ-ALA-D activity and an increase on brain and liver enzyme activity. DMPS caused an inhibition in renal δ-ALA-D activity, while animals that received a single dose of DMSA did not present δ-ALA-D activity of tissues modified. All three agents produced an increase in both liver and renal lipid peroxidation. Mice that received DMPS presented an increase in renal zinc concentration and a depletion of hepatic zinc occurred in mice administrated with BAL. Of all the toxic metals found in the environment and used in industry, cadmium occupies a special place because of the generally intractable nature of cadmium intoxication. Several lines of evidence indicate that reactive oxygen species are involved in cadmium-mediated tissue damage. Thus, it is believed that antioxidant should be one of the important components of an effective treatment of cadmium poisoning, as well, combined therapy with antioxidants and chelators can yield better therapeutic outcomes than isolated chelation therapy. Acute cadmium-intoxication caused inhibition of δ-ALA-D and superoxide dismutase (SOD) activities, reduction in ascorbic acid levels and increase of lipid peroxidation in mice testes. Also, an increase on plasmatic lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities was observed. DMSA, DMPS and diphenyl diselenide ($(PhSe)_2$) protected against the inhibitory effect of cadmium on δ-ALA-D activity and restored the increase of TBARS levels. However, these compounds alone or in combination, were unable to protect SOD activity and to improve ascorbic acid levels near to the normal value. The use of combined therapy (DMSA plus $(PhSe)_2$) not proved be better than the monotherapy, in improving the toxicological parameters evaluated in this model of testicular damage induced by cadmium.

On the other hand, the use of combined therapy (DMPS plus $(PhSe)_2$) proved to be better than the monotherapy in decreasing cadmium levels in testes and in ameliorating plasmatic AST level. Sub-chronic effects of cadmium-intoxication in mice caused inhibition of δ-ALA-D activity in liver, kidney and spleen and $(PhSe)_2$ therapy was effective in restoring enzyme activity in all tissues. Also, a reduction in ascorbic acid content by cadmium was observed in kidney and spleen, whereas $(PhSe)_2$ was only effective in improving this reduction in kidney. The therapy with this compound was effective in restoring an increase of TBARS levels caused by cadmium in liver and brain tissues. In general, the results of this study indicate that it is necessary to investigate the potential toxicity of the chelating agents, since chelators may be as harmful as the metals they chelate. In addition, $(PhSe)_2$ therapy in cadmium poisoning may be considered, as alone (antioxidant) as combined with DMPS and DMSA.

Key words: BAL, DMSA, DMPS, erythrocyte, ALA-D, cadmium, diphenyl diselenide, mice.

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ESQUEMAS

LISTA DE ABREVIATURAS

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

ALA – ácido 5’-aminolevulínico ou ácido delta-aminolevulínico

ALT – alanina aminotransferase

ANOVA – análise de variância

AST – aspartato aminotransferase

BAL – 2,3-dimercaptopropanol, dimercaprol

DMPS – ácido 2,3-dimercaptopropano 1-sulfônico

DMSA – ácido meso-2,3-dimercaptosuccínico

DMSO – dimetilsulfóxido

DP – desvio padrão

DTT – DL- ditiotreitol

EDTA – ácido etilenodiaminotetracético

PBG – porfobilinogênio

TCA – ácido tricloroacético

TFK – tampão fosfato de potássio

TBARS – espécies reativas ao ácido tiobarbitúrico

SOD – superóxido dismutase

SUMÁRIO

APRESENTAÇÃO

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos, os quais encontram-se no item **ARTIGOS CIENTÍFICOS**. As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigos e representam a íntegra deste estudo.

Os itens, **DISCUSSÃO E CONCLUSÕES**, encontrados no final desta tese, apresentam interpretações e comentários gerais sobre todos os artigos científicos contidos neste trabalho.

As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO**, **REVISÃO BIBLIOGRÁFICA, DISCUSSÃO e CONCLUSÕES** desta tese.

1 – INTRODUÇÃO

O uso terapêutico de agentes quelantes em casos de intoxicação por metais tóxicos vem sendo praticado há aproximadamente 40 anos. Estes compostos são utilizados clinicamente como antídotos para intoxicações agudas e crônicas por metais. Os agentes quelantes não somente aumentam a excreção, mas também reduzem a toxicidade dos metais por impedir a ligação destes a moléculas celulares alvo (Aposhian et al., 1995). Entretanto, o tratamento prolongado com agentes quelantes pode causar distúrbios hematopoiéticos (Flora e Kumar, 1993), desequilíbrio do metabolismo celular, síntese de DNA, RNA e proteína (Fischer et al., 1975), ou ainda alteração da homeostase dos elementos traços (Cantilena e Klaassen, 1982).

Um destes compostos, o 2,3-dimercaptopropanol (BAL), apresenta a capacidade de melhorar os efeitos deletérios causados pela intoxicação por metais. Entretanto, este composto apresenta um baixo índice terapêutico (Andersen, 1989) e produz neurotoxicidade (Pepin et al., 1995; Nogueira et al., 2000, 2001 a,b). Outros agentes quelantes tais como o ácido 2,3-dimercapto-1-propanosulfônico (DMPS, Dimaval®) e o ácido *meso*-2,3-dimercaptosuccínico (DMSA, Succimer®), são análogos estruturais do BAL mais hidrossolúveis, menos tóxicos e possivelmente, mais efetivos no tratamento das intoxicações (Aposhian et al., 1995). Entretanto, existem muitas questões referentes à utilização destes compostos que permanecem em discussão, incentivando a pesquisa nesta área.

Uma vez que 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).

Os metais endógenos são componentes essenciais de muitos sistemas enzimáticos. A δ-aminolevulinato desidratase (δ-ALA-D) é uma metaloenzima que requer íons zinco para sua atividade catalítica máxima (Jaffe et al., 1995). Esta enzima catalisa a condensação assimétrica de duas moléculas do ácido δ-aminolevulínico (δ-ALA), formando o porfobilinogênio, em um dos passos iniciais da biossíntese do heme (Gibson et al., 1955).

Além disso, a δ-ALA-D é uma enzima sulfidrílica e, diversos metais tais como o mercúrio (Rocha et al., 1995), o chumbo (Goering, 1993), assim como outros compostos capazes de oxidar os grupos sulfidrílicos da enzima podem modificar sua atividade (Emanuelli et al., 1996; Flora et al., 2002).

De todos os metais tóxicos encontrados no ambiente e utilizados industrialmente, o cádmio é um dos que apresenta maior interesse clínico, uma vez que as intoxicações por cádmio são geralmente intratáveis (Jones e Cherian, 1990). A intoxicação aguda por cádmio produz, primariamente injúria hepática e testicular, enquanto a exposição crônica produz dano renal e osteotoxicidade (Rikans et al., 2000). Muitas evidências indicam que as espécies reativas de oxigênio estão envolvidas na indução do dano tecidual pelo cádmio, o que causa estresse oxidativo como resultado do aumento na peroxidação lipídica e diminuição das defesas antioxidantes enzimáticas e não-enzimáticas (Koizumi e Li, 1992). Dessa forma, acredita-se que a utilização de antioxidantes, sozinhos ou em associação com os agentes quelantes, seria uma alternativa mais eficaz para um tratamento das intoxicações por cádmio (Pande et al., 2001).

O conceito de que moléculas contendo selênio podem ser melhores nucleófilos (e portanto antioxidantes) do que os antioxidantes clássicos tem levado ao desenvolvimento de compostos orgânicos de selênio (Arteel e Sies, 2001). Vários relatos foram publicados sobre compostos de selênio que apresentam atividade como miméticos da glutationa peroxidase. Além disso, um estudo recente relatou que disselenetos de diarila apresentaram atividade antioxidante em camundongos e o disseleneto de difenila ($(PhSe)_2$) demonstrou ser mais ativo como mimético da glutationa peroxidase (Meotti et al., 2004) e menos tóxico em roedores do que o ebselen (Nogueira et al., 2003a; Meotti et al., 2003).

Considerando os aspectos acima mencionados, o presente estudo visa abordar dois aspectos principais: (1) estudar o efeito *per se* dos agentes quelantes sulfidrílicos (BAL, DMPS e DMSA), a fim de verificar um possível efeito tóxico destes compostos e (2) avaliar o efeito dos mesmos isolados ou em associação com um antioxidante ($(PhSe)_2$) em modelos de intoxicação por cádmio em camundongos.

2 – REVISÃO BIBLIOGRÁFICA

2.1 - Agentes Quelantes Sulfidrílicos

O aumento do uso industrial de metais como o berílio, o cádmio, o cobre, o chumbo, o magnésio e o níquel inevitavelmente resultaram em um ambiente no qual as intoxicações crônicas não são raras. Conseqüentemente, os riscos ocupacionais e ambientais para a saúde humana originados da exposição ao metal são uma preocupação (Domingo, 1998).

A terapia que utiliza os agentes quelantes tem sido à base do tratamento médico de intoxicações por metal desde a década de 40, sendo os mesmos utilizados clinicamente como antídotos para intoxicações agudas e crônicas (Domingo, 1995). Estes compostos atuam aumentando a excreção de elementos tóxicos, como o arsênio, o cádmio, o chumbo e o mercúrio, os quais são bem conhecidos pela toxicidade que desenvolvem. Além disso, eles também diminuem a toxicidade do metal por prevenir a ligação destes a moléculas celulares alvo (Domingo, 1995). Entretanto, existem, ainda, muitas questões referentes à utilização destes compostos que permanecem em discussão, incentivando a pesquisa nesta área.

2.1.1 – Histórico

2.1.1.1 - 2,3-dimercaptopropanol ou dimercaprol (BAL)

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).

Após estes achados, Thompson testou os efeitos do BAL no seu próprio antebraço e relatou a eficácia deste composto para o uso em humanos. A partir destes estudos, o 2,3-dimercaptopropanol tornou-se conhecido como dimercaprol ou simplesmente BAL (British Anti-Lewisite) (Stocken e Thompson, 1946).

No final de 1947 intensificaram-se os estudos a respeito do BAL, especialmente na Inglaterra e nos EUA. Embora ele tenha sido desenvolvido como um antídoto para intoxicações por arsênio, este composto passou a ser indicado para o tratamento de intoxicações agudas por outros elementos tóxicos, como o mercúrio, o chumbo e o cádmio (Stocken, 1947; Klaassen, 1990; 1996; Emanuelli et al., 1996).

2.1.2 - Estrutura Química e Indicações Clínicas

O BAL é um composto sulfidrílico, líquido, denso e incolor (Aposhian et al., 1983). Devido à sua lipossolubilidade e instabilidade em soluções aquosas, ele deve ser administrado dissolvido em óleo vegetal, através de injeção intramuscular (Klaassen 1996).

A lipo- ou hidrossolubilidade dos agentes quelantes é característica importante e pode limitar a sua utilização e eficácia. Compostos lipofílicos, como o BAL, podem facilmente atravessar a membrana celular e atingir os espaços intracelulares (Andersen, 1989).

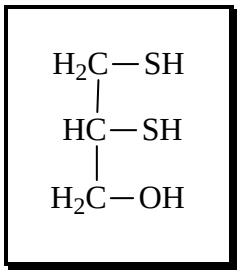

Figura 1- Estrutura do 2,3- dimercaptopropanol (BAL).

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 sulfidrílicos (-SH), incluindo o BAL, a D-penicilamina (Schwartz et al., 1992; Klassen, 1996) e o ditiotreitol (DTT) (Suzuki et al., 1994). Estes compostos ditiólicos possuem a capacidade de complexar este metal pesado e aumentar a velocidade de excreção renal e biliar (Jugo, 1980; Kojima et al., 1989; Shimada et al., 1993).

Gilman e colaboradores (1946) propuseram que os metais pesados são tóxicos aos sistemas biológicos por formarem mercaptídeos reversíveis, a partir de grupos -SH de moléculas protéicas, tais como enzimas. Estes autores também postularam uma hipótese para explicar o mecanismo pelo qual os ditióis são capazes de reativar sistemas enzimáticos e exercer benefícios no tratamento de intoxicações com metais pesados. Segundo esta hipótese, o uso de quelantes ditiólicos proporcionaria a formação de mercaptídeos de baixa dissociabilidade, o que reverteria, efetivamente, a ligação dos metais pesados com sistemas proteicos sensíveis a estes (Gilman et al., 1946).

As primeiras aplicações clínicas do BAL foram descritas para o tratamento de dermatites, decorrentes do uso terapêutico de compostos orgânicos contendo arsênio, usual na época para o tratamento de pacientes com sífilis (Longcope et al., 1946). Outras indicações clínicas deste composto referem-se a sua utilização em formulações farmacêuticas. O Melarsoprol®, uma preparação farmacêutica que contém BAL, é indicado no tratamento da doença do sono causada pelo *Trypanosoma brucei gambiense*. Este composto foi testado em camundongos infectados e demonstrou eficácia em eliminar os tripanossomas circulantes e inclusive do sistema nervoso central (Jennings et al., 1996a; Jennings et al., 1996b; Pepin et al., 1995).

2.1.3 – Toxicidade

A toxicidade dos agentes quelantes pode limitar o seu valor terapêutico. Esta é a principal razão para o declínio do uso do BAL e o aumento do uso de outros quelantes ditiólicos, tais como o DMPS (ácido 2,3-dimercapto-1-propanosulfônico) e o DMSA (ácido *meso* 2,3-dimercaptosuccínico), derivados mais hidrossolúveis e possivelmente menos tóxicos deste composto (Andersen, 1989).

Dessa forma, devido às características estruturais e de solubilidade, a utilização do BAL apresenta várias desvantagens e, portanto tem sido questionada. Em torno de 50% dos pacientes tratados com este composto apresentam efeitos colaterais (Klaassen, 1985; Aposhian et al., 1992), tais como, aumento da pressão sistólica e diastólica, taquicardia, naúseas, vômitos e dor de cabeça (Aposhian, 1983). Além disso, o BAL apresenta um baixo índice terapêutico, uma vez que a sua dose terapêutica efetiva é muito próxima da dose letal (Chilsolm, 1970; Andersen, 1989; Kojima et al., 1989). A DL_{50} para camundongos é 0,73 $mmol/kg^{-1}$ (Zvirblis e Ellin, 1976; Cantilena e Klaassen, 1981). Este composto deve ser injetado por via intramuscular, o que dificulta sua utilização (Aposhian et al., 1984).

Devido à sua lipossolubilidade, o BAL pode atravessar a membrana celular e atingir os espaços intracelulares (Andersen, 1989), sendo completamente contra-indicado em intoxicações agudas por cloreto de cádmio (Tepperman, 1947). A formação do complexo metal-BAL aumenta a redistribuição tendo como conseqüência o acréscimo da deposição de cádmio no cérebro. A redistribuição de metais dos órgãos periféricos para o cérebro, após o tratamento com BAL, tem sido também descrito para outros metais, incluindo o arsênio, o metil mercúrio (Hoover e Aposhian, 1983; Emanuelli et al.,1996), o mercúrio inorgânico (Aaseth et al., 1995) e o chumbo (Cory-Slechta et al., 1987).

Como conseqüência, o BAL pode atingir níveis tóxicos em pacientes com nefropatia ou falência renal, situação comum em pacientes severamente intoxicados com cloreto de mercúrio (Clarkson, 1990), além de induzir sintomas clínicos tais como ansiedade, hiperatividade, hiperflexia e convulsões (Toet et al., 1994). Entretanto, em intoxicações agudas com arsênio o tratamento com BAL continua sendo fundamental (Muckter et al., 1997).

Além disso, modelos experimentais mostraram que a administração de doses elevadas deste composto produz convulsões que culminam com a morte do animal, sugerindo ainda, que a modulação de receptores GABAérgicos, glutamatérgicos e canais iônicos estejam envolvidos no mecanismo de neurotoxicidade induzida pelo BAL (Nogueira et al., 2000). Estudos do nosso grupo demonstraram que a toxicidade induzida pelo BAL pode estar relacionada com a inibição da captação e o aumento da liberação de glutamato em sinaptossomas de ratos (Nogueira et al., 2001), reafirmando o potencial neurotóxico do BAL.

Mesmo que diversos estudos tenham demonstrado que o BAL é capaz de induzir toxicidade, no Brasil, este agente quelante de metais tóxicos está na "Lista de medicamentos prioritários para registro de genéricos de 2002" da Agência Nacional de Vigilância Sanitária (ANVISA).

2.1.4 - Análogos Estruturais do BAL

A estratégia terapêutica para tratar a intoxicação com metais envolve a administração crônica de agentes quelantes (Cantilena e Klaassen, 1982). Entretanto, um dos maiores problemas relacionados à eficiência dos agentes quelantes em intoxicações crônicas com metais pesados é a capacidade dos metais em induzir a síntese de proteínas de baixo peso molecular, metalotioneínas e a conseqüente ligação intracelular específica e de alta afinidade dos metais a estas proteínas (Mehta e Flora, 2001).

Em 1990, Jones e Cherian descreveram as características ideais para que um agente quelante fosse efetivo no tratamento de intoxicações com metais pesados. Entre elas, a facilidade de entrar na célula, quelar os metais ligados a metalotioneínas e aumentar a excreção destes. Tudo isso, sem causar danos aos tecidos e sem aumentar os níveis do metal em outros orgãos críticos, tais como o cérebro.

Uma vez que a utilização do BAL apresenta diversas limitações e devido à sua toxicidade, outros agentes quelantes, potencialmente menos tóxicos, têm sido investigados (Keith et al., 1997).

Derivados estruturais do BAL, o DMPS (ácido 2,3-dimercapto-1-propanosulfônico) e o DMSA (ácido *meso* 2,3-dimercaptosuccínico) (Figuras 2 e 3, respectivamente), são compostos que apresentam dois grupos sulfidrílicos (-SH) vicinais e caracterizam-se pela maior solubilidade em água (Nadig et al., 1985) e limitada solubilidade lipídica (Aposhian et al., 1983).

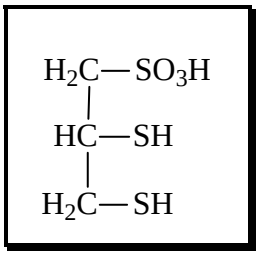

Figura 2- Estrutura do ácido 2,3-dimercapto-1-propanosulfônico (DMPS).

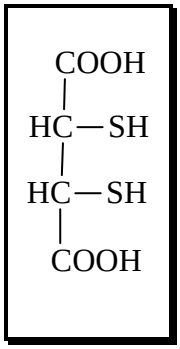

Figura 3- Estrutura ácido *meso* 2,3- dimercaptosuccínico (DMSA).

Várias são as vantagens do DMPS e do DMSA, em relação ao BAL. Apresentam-se como sólidos cristalinos e estáveis (Aposhian et al., 1992) e são facilmente administrados por via oral (Aposhian et al., 1996). A DL_{50} em camundongos é 5,22 $mmol.kg^{-1}$ para o DMPS (Aposhian et al., 1981) e 16,5 $mmol.kg^{-1}$ para o DMSA (Friedheim et al., 1976), portanto são maiores que a DL_{50} para o BAL (0,73 $mmol.kg^{-1}$). Devido a estas características, estes compostos são considerados menos tóxicos que o BAL (Aposhian et al., 1992).

O DMPS 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). A eficácia do DMSA, Succimer®, foi originalmente descrita por pesquisadores chineses (Wang et al., 1965). Este composto foi aprovado em 1991 nos EUA pelo FDA (Food and Drug Admistration) apenas para o tratamento de intoxicações agudas e crônicas por chumbo em crianças com níveis sanguíneos deste metal maiores que 45 μg/dl (U.S. Department of Health and Human Services, 1991). Sendo assim, o uso dessas drogas continua sendo experimental (Nielsen e Andersen, 1991; Aposhian et al., 1995).

O DMPS foi descrito como uma droga efetiva no tratamento de intoxicações por mercúrio (Kostygou, 1958). Segundo diversos relatos, é uma alternativa segura e eficaz para substituir 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). Entretanto, este composto parece não ser efetivo no tratamento de intoxicações por chumbo (Chisolm, 1992) e cádmio (Jones e Cherian, 1990), embora não cause redistribuição de chumbo para o cérebro, em ratos (Aposhian et al., 1996).

A eficácia do DMSA foi originalmente descrita por pesquisadores chineses (Wang et al., 1965). É um quelante, oralmente efetivo, que pode ser usado no tratamento de crianças intoxicadas com chumbo, desde que apresentem níveis sangüíneos superiores a 45 μg/dL deste metal (Graziano et al., 1978; 1978a; Friedheim et al., 1976; Pappas et al., 1995).

O tratamento terapêutico padrão recomendado para crianças com encefalopatia induzida por chumbo, consta da associação de BAL e EDTA (Chisolm, 1992). Todavia, o DMSA parece ser efetivo em remover chumbo do cérebro (Cory-Slechta, 1988). Portanto, quando comparado a outros quelantes, tais como BAL, EDTA e D-penicilamina, o DMSA é menos tóxico e é efetivo em reduzir a concentração sangüínea e aumentar a excreção urinária de chumbo em crianças (Graziano et al., 1988; 1992; Chisolm, 1990) e animais (Cory-Slechta, 1988; Flora et al., 1995; Gong e Evans, 1997). Outros estudos indicam que o DMSA pode ser mais efetivo que o BAL em proteger da nefrotoxicidade induzida por metil mercúrio (De LaTorre et al, 1998).

Estudos clínicos estabeleceram que o DMSA é uma droga eficaz e segura e têm sugerido este composto como o quelante de preferência, em casos de intoxicações com metais (Miller, 1998). Contudo, o assunto apresenta algumas controvérsias; isto é, segundo alguns autores o DMSA apresenta a desvantagem de causar a redistribuição de chumbo dos orgãos periféricos para o cérebro (Cory-Slechta, 1988; Flora et al., 1995; Jones et al., 1994) e causar a eliminação de metais fisiologicamente essenciais (Graziano et al., 1978a). Porém, experiências recentes claramente relatam a distribuição extracelular do *meso*-DMSA (Aposhian e Aposhian, 1990) e sua pouca eficiência em intoxicações com cádmio, visto que não retira o metal ligado a metalotioneínas (Aposhian et al., 1992).

Segundo Aposhian e colaboradores (1992), o DMSA é três vezes menos tóxico que o DMPS. Entretanto, Aposhian e Aposhian (1990) sugerem precaução no uso do DMSA para gestantes, uma vez que apresenta efeitos embriotóxicos, em camundongos, quando administrado em altas doses.

O DMPS parece ser mais efetivo para remover o mercúrio em ratos (Planas-Bohne, 1981), além de ser melhor agente terapêutico, pois mobiliza mercúrio mais rápido que o DMSA (Buchet e Lauwerys, 1989).

Recentemente, o DMPS e o DMSA têm sido utilizados para tratar intoxicações por mercúrio e chumbo e mostraram-se efetivos em estudos clínicos (Aposhian e Aposhian, 1990; Graziano et al., 1988; Toet et al., 1994).

Considerando o descrito acima, permanece em aberto a questão a respeito de qual seria a droga de escolha, em casos de intoxicação com metais pesados (Muckter et al., 1997).

2.2 – Efeitos Tóxicos dos Quelantes Sobre a Enzima Delta-Aminolevulinato Desidratase (δ-ALA-D)

Além dos possíveis efeitos tóxicos que os quelantes podem apresentar durante o tratamento de intoxicações por diversos metais tóxicos, sabe-se também que os agentes quelantes ditiólicos, em especial o BAL, apresentam atividade inibitória *per se* sobre a enzima δ-aminolevulinato desidratase (δ-ALA-D) de rim (Emanuelli et al., 1996), assim como o DMPS e o DMSA sobre a enzima hepática (Nogueira et al., 2003b) de camundongos.

2.2.1 – Histórico e função da δ-ALA-D

Isolada nos anos 50 (Gibson et al., 1955), a metalo-proteína citoplasmática δ-aminolevulinato desidratase (δ-ALA-D, E.C. 4.2.1.24), também conhecida como porfobilinogênio sintase ou 5-aminolevulinato hidroliase é a enzima que catalisa a condensação assimétrica de duas moléculas de ácido delta-aminolevulínico (ácido 5-aminolevulínico, ALA), formando o composto monopirrólico porfobilinogênio (PBG), com a perda de 2 moléculas de água (Shemin, 1976) (figura 4).

Esta reação catalisada pela δ-ALA-D faz parte da rota biossintética dos compostos tetrapirrólicos (corrinas, bilinas, clorofilas e hemes), sendo esta via biossintética semelhante em bactérias, vegetais e animais (Rodrigues, 1989). Nos mamíferos, os tecidos que apresentam maior atividade desta enzima são o hepático, o renal e os tecidos hematopoiéticos (Gibson et al., 1955).

Os compostos tetrapirrólicos têm importância metabólica baseada, principalmente, na sua função como grupos prostéticos de proteínas. O heme (ferroprotoporfirina), por exemplo, 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).

Guo et.al., (1994) demonstraram que a enzima δ-ALA-D é idêntica ao inibidor de proteossoma de 240-Kda (CF-2) Estes achados conferem a esta enzima uma importância adicional, uma vez que os proteossomas atuam na degradação de proteínas anormais, fatores de transcrição, oncoproteínas, bem como no processamento de antígenos (Wlodawer, 1995).

2.2.2 – Características estruturais e cinéticas

A δ-ALA-D é uma enzima de natureza sulfidrílica (Shemin, 1976; Bevan et al., 1980), sendo inibida por agentes bloqueadores de grupos sulfidrílicos, tais como N-etilmaleimida, iodoacetato (Jordan et al., 1976; Barnard et al., 1977), para-

cloromercúriobenzoato, monoiodoacetamida e DTNB (Barreiro, 1967; Barnard et al., 1977; Tamai et al., 1979) e por metais pesados que possuam elevada afinidade por grupamentos sulfidrílicos, como o chumbo, o cobre e o mercúrio (Gibson et al., 1955; Rocha et al., 1995; Emanuelli et al., 1996). A imensa maioria das enzimas δ-ALA-D isoladas até o momento requer um íon metálico bivalente para estarem ativas, sendo normalmente inibidas por EDTA. Apesar do alto grau de similaridade entre os genes da δ-ALA-D provenientes de diversos organismos, as enzimas, dependendo de sua fonte, requerem metais diferentes para sua ativação.

A δ-ALA-D, de animais, leveduras e bactérias, é dependente de zinco (Chen e Neilands, 1973; Finelli et al., 1974), sendo também demonstrado que resíduos de cisteína da proteína estão envolvidos na união deste metal (Dent et al., 1990; Spencer e Jordan, 1994). Por outro lado, a enzima proveniente de vegetais, apesar de possuir uma similaridade de 35-50 % com a de outras fontes, não requer zinco, mas sim magnésio (Shibata e Ochiai, 1977; Tamai et al., 1979). A região rica em cisteína presente na enzima de origem animal, e que corresponde à região que supostamente liga zinco, é substituída na enzima de vegetais por uma região rica em aspartato, que caracterizaria o sítio para a união do magnésio (Boese et al., 1991; Schaumburg et al., 1991).

A curva de velocidade da catálise em função da concentração de substrato, para a δ-ALA-D de origem animal, apresenta um perfil sigmóide, denotando o caráter alostérico da enzima (Chinarro et al., 1983), enquanto que a δ-ALA-D da *Saccharomyces cerevisiae* demonstra cinética tipicamente Michaeliana (Borralho et al., 1990).

O pH ótimo para a determinação da atividade da δ-ALA-D varia de 6,2 a 9,5, de acordo com a sua fonte (Barreiro et al., 1967), mas a enzima de origem animal apresenta valores mais baixos de pH ótimo (Gibson et al., 1955) que a de origem vegetal (Shibata e Ochiai, 1977; Tamai et al., 1979).

2.2.3 - Mecanismo catalítico

Evidências sugerem que o sítio ativo da enzima seja composto por resíduos de cisteína, dois átomos de zinco, um resíduo de histidina, um resíduo de lisina e alguns resíduos de aminoácidos hidrofóbicos (Tsukamoto et al., 1979; Jaffe et al., 1994).

A δ-ALA-D possui 8 subunidades, no entanto, apenas metade das subunidades parece estar envolvida na catálise (Shemin, 1976; Jaffe e Hanes, 1986), ocorrendo o fenômeno de "half-site reactivity" (Seydoux et al., 1974).

Sabe-se que 3 tipos diferentes de aminoácidos são essenciais para a atividade da δ-ALA-D:

I – um resíduo de lisina ao qual se liga a primeira molécula de substrato, através de uma base de Schiff (Gibbs e Jordan, 1986);

II – um resíduo de histidina, o qual pode sofrer fotooxidação, reduzindo tanto a atividade enzimática quanto à ligação ao zinco (Tsukamoto et al., 1979). Este resíduo poderia participar no mecanismo de transferência de prótons do meio aquoso ao sítio ativo hidrofóbico;

III – pelo menos dois resíduos de cisteína, os quais devem estar reduzidos para que a enzima apresente atividade máxima (Chen e Neilands, 1976). Estes resíduos são altamente reativos, podendo formar uma ponte dissulfeto em presença de ar, formar mercaptídios por reação com metais pesados ou serem modificados por agentes químicos. A oxidação desses resíduos leva à inativação com concomitante perda do zinco ligado (Tsukamoto et al., 1979).

Basicamente, o mecanismo proposto para a síntese do porfobilinogênio é o de que um resíduo lisil do sítio ativo da δ-ALA-D forma uma base de Schiff com a primeira molécula do substrato (ALA), originando a cadeia lateral P (cadeia propiônica), enquanto uma segunda molécula do substrato dá origem à cadeia lateral A (acética) do porfobilinogênio (Castelfranco e Beable, 1983) (figura 4).

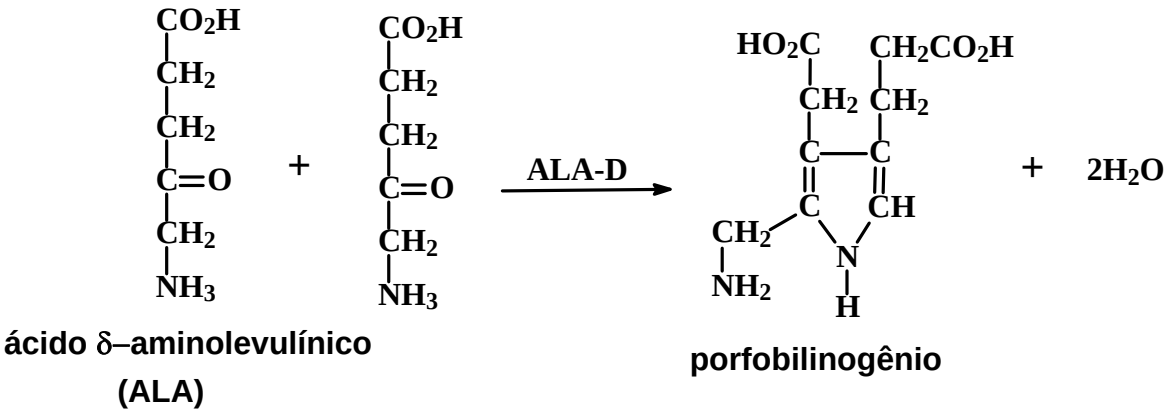

Figura 4: Condensação assimétrica de 2 moléculas do ALA, catalisada pela enzima δ-aminolevulinato desidratase.

2.2.4 – A função dos metais bivalentes (Zn^{2+} e Mg^{2+}) na δ-ALA-D

A δ-ALA-D de mamíferos é inibida por quelantes como EDTA e 1,10-fenantrolina (Chen e Neilands, 1976; Sommer e Beyersmann, 1984), sendo esta inibição revertida pela adição de zinco (Bevan et al., 1980). Isto mostra que o zinco faz parte da estrutura da enzima e, possivelmente, tenha um papel fundamental no seu mecanismo catalítico. Entretanto, o papel do zinco na atividade da δ-ALA-D não está, ainda, completamente elucidado. Algumas evidências sugerem uma função catalítica direta, enquanto outras apontam para uma função estrutural do zinco na enzima, ou ambas (Tsukamoto et al., 1979; Dent et al., 1990; Spencer e Jordan, 1995).

Após a remoção do zinco pelo EDTA, os grupos –SH da enzima são facilmente oxidados, com concomitante perda da atividade enzimática. A apoenzima oxidada obtida então, apenas incorporará zinco novamente na presença de um ativador sulfidrílico (Tsukamoto et al., 1979; Bevan et al., 1980).

Existe uma controvérsia quanto ao número de átomos de zinco que seriam necessários para a atividade máxima da δ-ALA-D de mamíferos, com este número variando entre 4 e 8 (Shemin, 1976; Bevan et al., 1980; Tsukamoto et al., 1980). Quando ensaiada na presença de altas concentrações de um agente redutor, a enzima requer apenas 4 íons zinco por octâmero

para apresentar atividade máxima (Bevan et al., 1980). Estes quatro íons zinco são denominados catalíticos.

Sabe-se da existência de 2 sítios estruturalmente distintos para a ligação do zinco na δ-ALA-D bovina (Dent et al., 1990). O primeiro deles seria composto por 5 ligantes: 2 ou 3 histidinas, 1 ou nenhum oxigênio de um grupo como tirosina ou uma molécula solvente, 1 tirosina ou aspartato e 1 –SH da cisteína. Este sítio estaria envolvido na ligação dos 4 íons zinco essenciais para a completa ativação da δ-ALA-D, os quais têm sido referidos como catalíticos. Este zinco catalítico parece ser importante para a união da segunda molécula de substrato, para a formação da primeira ligação entre as duas moléculas de ALA (ligação carbono-nitrogênio) e para a união do produto (Jaffe et al., 1992; Spencer e Jordan, 1994). O segundo sítio seria composto por 4 resíduos de cisteína e estaria envolvido na ligação dos outros 4 átomos de zinco não essenciais, os quais têm sido referidos como estruturais.

2.2.5 – Importância toxicológica

A enzima δ-ALA-D, devido a sua natureza sulfidrílica, pode ser inibida na presença de elementos como mercúrio (Rocha et al., 1993, 1995; Emanuelli et al., 1996), chumbo (Rodrigues et al., 1989, 1996) e outros que tenham alta afinidade por grupos sulfidrilicos. Esta enzima também é inibida por compostos orgânicos contendo telúrio e selênio (Barbosa et al., 1998; Maciel et al., 2000; Farina et al., 2001), os quais oxidam grupos sulfidrilicos.

Uma inibição da atividade da δ-ALA-D, além de prejudicar a rota biossintética da molécula do heme pode resultar em um acúmulo do substrato ALA no fígado e no sangue, com conseqüente aumento na excreção urinária do mesmo (Sassa et al., 1989).

Deficiências na via de biossíntese do heme são conhecidas como porfirias. A porfiria por deficiência da δ-ALA-D, também conhecida por “porfiria aguda”, pode ser adquirida, como ocorre em casos de intoxicação por chumbo (Granick, 1978), ou pode resultar de uma doença recessiva autossômica rara, onde devido a uma quase completa ausência de atividade da enzima, os pacientes excretam altas quantidades de ALA urinário (Sassa, 1989).

Entretanto esta forma de porfiria é muito rara, havendo apenas quatro casos documentados no mundo (Kappas et al., 1995).

A inibição da δ-ALA-D também parece estar relacionada com a patogênese da tirosinemia hereditária (Rank et al., 1991). Nesta doença, a succinilacetona (análogo estrutural do substrato ALA) é acumulada devido a uma deficiência genética na via de degradação da tirosina, inibindo competitivamente a δ-ALA-D (Sassa e Kappas, 1983).

Numerosas observações clínicas têm associado o acúmulo de ALA com a patogênese das porfirias agudas (Kappas et al., 1995). Estas evidências são reforçadas pelo fato de pacientes com tirosinemia hereditária do tipo I ou intoxicações com chumbo, doenças associadas ao acúmulo de ALA, apresentarem sintomas semelhantes àqueles dos pacientes com porfirias agudas hereditárias (Sassa e Kappas, 1983; Klaassen, 1996).

Além das observações clínicas, evidências experimentais reforçam a hipótese de que o ALA esteja envolvido na patogênese dessas desordens.

Ao nível neuroquímico, 1 μM de ALA é capaz de inibir a liberação do neurotransmissor ácido-γ-aminobutírico (GABA), assim como a sua ligação nas membranas sinápticas (Brennan et al., 1979; 1980), tanto em ratos quanto em humanos (Emanuelli et al., 2001a). O ALA também demonstrou poder inibitório sobre a atividade da Na^+, K^+, ATPase de cérebro e eritrócitos de coelhos (Becker et al., 1971), além de diminuir a velocidade de condução de impulsos nervosos motores em camundongos (Cutler et al., 1979). *In vivo*, a administração intraestriatal de ALA induz convulsões e assimetria corporal (Emanuelli et al., 2000).

O acúmulo de ALA também está relacionado com a superprodução de espécies reativas de oxigênio (Bechara et al., 1993). Tem sido demonstrado que a oxidação do ALA, in vitro, pode provocar lipoperoxidação (Oteiza et al., 1994), lesões em mitocôndrias hepáticas (Hermes-Lima et al., 1991), liberação de ferro da ferritina (Oteiza et al., 1994), lesões no DNA e inibição da adenilato ciclase cerebral (Emanuelli et al., 2001b).

2.3 – Cádmio

A exposição das populações humanas a uma variedade de metais tóxicos é um problema de saúde pública (Goyer, 1996). De todos os metais tóxicos encontrados no ambiente e utilizados industrialmente, o cádmio é um dos que apresenta maior interesse clínico, uma vez que as intoxicações por cádmio são geralmente intratáveis (Jones e Cherian, 1990).

A toxicidade do cádmio, tanto em animais experimentais quanto em humanos, é influenciada por um grande número de fatores, tais como a via de administração, a dose, a forma química do metal, a duração da exposição, a idade dos animais experimentais, etc. (Casalino et al., 1997). A intoxicação aguda por cádmio produz primariamente injúria hepática e testicular, enquanto a exposição crônica produz dano renal e osteotoxicidade (Rikans et al., 2000). Dessa forma, sob condições de exposição mais prolongada ao cádmio, este metal se deposita primariamente no fígado, onde ele induz e se liga às metalotioneínas (MT) (Shaikh and Lucis, 1972), podendo também causar efeitos hepatotóxicos. Com o tempo, o complexo CdMT hepático é lentamente liberado na circulação (Tohyama and Shaikh, 1981) e posteriormente, após filtração glomerular, este complexo é degradado e os íons cádmio liberados se ligam a metalotioneínas renais pré-existentes ou àquelas recentemente sintetizadas (Cherian, 1978). Quando a quantidade de cádmio presente no córtex renal excede a capacidade de ligação às metalotioneínas, este cádmio não ligado a MT é capaz de causar nefrotoxicidade (Nomiyama and Nomiyama, 1986), provavelmente pela geração de radicais livres (Hassoun and Stohs, 1996).

Muitas evidências indicam que as espécies reativas de oxigênio estão envolvidas na indução do dano tecidual pelo cádmio, o que causa estresse oxidativo como resultado do aumento na peroxidação lipídica e diminuição das defesas antioxidantes enzimáticas e não-enzimáticas (Koizumi and Li, 1992). Dessa forma, a prevenção e a intervenção terapêutica na intoxicação pelo cádmio podem ser conduzidas de duas maneiras: (1) a utilização de quelantes a fim de remover o cádmio ligado à metalotioneína localizado intracelularmente, principalmente no fígado e no rim (Goyer et al., 1995; Andersen, 1999); e (2) a eliminação dos radicais livres por antioxidantes e sistemas de defesa enzimáticos (Farris, 1991).

Sendo assim, acredita-se que a utilização de antioxidantes, sozinhos ou em associação com os agentes quelantes, seria uma alternativa mais eficaz para o tratamento das intoxicações por cádmio (Flora, 1999; Pande et al., 2001; Tandon et al., 2003). De fato, estes autores obtiveram bons resultados com estas terapias, principalmente, proteção contra o aumento da peroxidação lipídica, recuperação dos sistemas antioxidantes enzimáticos e ainda, redução do conteúdo de cádmio tecidual. Entretanto, não há até o momento um consenso com relação ao tratamento mais seguro e eficaz das intoxicações agudas e crônicas por cádmio, o que tem incentivado a pesquisa de terapias alternativas, em especial de compostos antioxidantes, administrados sozinhos ou associados aos quelantes ditiólicos (DMSA, DMPS) com efeitos benéficos previamente comprovados.

2.4 - Organocalcogênios

A partir da década de 30, os organocalcogênios têm sido alvo de interesse para os químicos orgânicos em virtude da descoberta de aplicações sintéticas (Petragnani et al., 1976; Comasseto, 1983) e de propriedades biológicas desses compostos (Parnham e Graf, 1991; Kanda et al., 1999), que são 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., 2003c).

2.4.1 - Selênio

Esse elemento químico foi descoberto em 1817, pelo químico sueco J. J. Berzelius. O selênio é um elemento 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).

Os selenóis (R-SeH) são as formas correspondentes aos tióis (R-SH), onde ocorre a substituição do átomo de enxofre pelo átomo de selênio (Klayman e Günther,1973).

2.4.1.1 - Atividade biológica

O selênio é um elemento traço, 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. 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 (Food and Nutrition Board, 1989).

O selênio apresenta um grande número de funções biológicas, sendo a mais importante como antioxidante.

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 selenoproteina P (Ursini et al., 1990). A atividade redox do selênio tem fundamental importância para o sítio catalítico dessas enzimas.

2.4.1.2 – Disseleneto de Difenila ($(PhSe)_2$)

O conceito de que moléculas contendo selênio podem ser melhores nucleófilos (e portanto antioxidantes) do que os antioxidantes clássicos tem levado ao desenvolvimento de compostos orgânicos de selênio sintéticos (Arteel e Sies, 2001).

Vários relatos foram publicados sobre compostos de selênio que apresentam atividade como miméticos da glutationa peroxidase. Além disso, um estudo recente relatou que disselenetos de diarila apresentaram atividade antioxidante em camundongos e o disseleneto de difenila ($(PhSe)_2$) demontrou ser mais ativo como mimético da glutationa peroxidase (Meotti et al., 2004) e menos tóxico em roedores do que o ebselen (Nogueira et al. 2003a; Meotti et al. 2003). Além disso, nosso grupo de estudo tem demonstrado que o disseleneto de difenila tem outras propriedades farmacológicas, tais como efeitos anti-úlcera (Savegnago et al., 2005), anti-inflamatório e antinociceptivo (Nogueira et al., 2003c, Zasso et al., 2005).

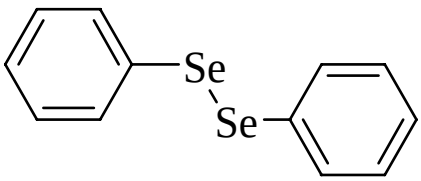

Figura 5 – Disseleneto de Difenila ($(PhSe)_2$)

3. OBJETIVOS

Os agentes quelantes sulfidrílicos, embora sejam efetivos em remover os metais tóxicos nas intoxicações, apresentam alguns efeitos tóxicos *per se*. Neste contexto, trabalhos prévios do nosso grupo demonstraram que os agentes quelantes ditiólicos, em especial o BAL, apresentam atividade inibitória *per se* sobre a enzima δ-aminolevulinato desidratase (δ-ALA-D) de rim (Emanuelli et al., 1996), assim como o DMPS e o DMSA sobre a enzima hepática (Nogueira et al., 2003). Dessa forma, este trabalho visa abordar dois aspectos principais: (1) estudar o efeito *per se* dos agentes quelantes sulfidrílicos (BAL, DMPS e DMSA), a fim de verificar um possível efeito tóxico destes compostos sobre a δ-ALA-D de eritrócitos humanos *in vitro* e sobre alguns parâmetros toxicológicos em camundongos *ex vivo* e (2) avaliar o efeito destes compostos sozinhos ou em associação com um antioxidante, $(PhSe)_2$, em modelos de intoxicação aguda e sub-crônica por cádmio em camundongos.

4- ARTIGOS CIENTÍFICOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos científicos, os quais encontram-se aqui organizados. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigos. Os artigos estão dispostos da mesma forma que foram publicados na edição das revistas científicas (**Artigos 1, 2, 3**) ou aceitos, que é o caso dos **Artigos 4 e 5 (ainda não publicados)**.

4.1 – Efeito *per se* dos agentes quelantes sulfidrílicos (BAL, DMPS e DMSA) sobre parâmetros toxicológicos: Estudos *in vitro* e *ex vivo*

4.1.1 - **Artigo 1**

2,3- DIMERCAPTOPROPANOL, 2,3-DIMERCAPTOPROPANE-1-SULFONIC ACID AND *MESO*-2, 3-DIMERCAPTOSUCCINIC ACID INHIBIT δ-AMINOLEVULINATE DEHYDRATASE FROM HUMAN ERYTHROCYTES *IN VITRO*

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2,3-Dimercaptopropanol, 2,3-dimercaptopropane-1-sulfonic acid, and *meso*-2,3-dimercaptosuccinic acid inhibit δ-aminolevulinate dehydratase from human erythrocytes in vitro

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Abstract

The effects of dithiol chelating agents *meso*-2,3-dimercaptosuccinic acid (DMSA), 2,3-dimercaptopropane-1-sulfonic acid (DMPS), and 2,3-dimercaptopropanol (BAL) on δ-aminolevulinate dehydratase (δ-ALA-D) from human erythrocytes were evaluated. Furthermore, possible protective effects of zinc chloride ($ZnCl_2$), dithiothreitol (DTT), and cysteine were studied. δ-ALA-D activity from human erythrocytes was inhibited by dithiol chelating agents in a concentration-dependent manner. Cysteine, at all concentrations tested, did not protect the inhibitory effect of 1 and 4 mM DMPS and DMSA, but protected 1 mM BAL inhibition. Dithiotreitol was able to protect the inhibition caused by 1 mM BAL (28%), DMPS (56%), and DMSA (40%) in a concentration-dependent manner. Zinc chloride protected and restored 1 mM BAL inhibitory effect on δ-ALA-D. Zinc chloride at 500 μM and 1 mM, respectively, protected inhibitory effects of DMPS and DMSA (1 and 4 mM), but did not reverse its effects. The preincubation of dithiol chelating agents with enzyme demonstrated that DMSA was the most potent δ-ALA-D inhibitor of human erythrocytes. These data are in agreement with δ-ALA-D activity from purified enzyme. $ZnCl_2$ (1 μM) added, in the reaction mixture, increased enzyme activity and DTT (100 μM) totally restored the enzyme activity for all chelating agents tested.
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Keywords: BAL; DMSA; DMPS; Erythrocyte; ALA-D

1. Introduction

Several metals are known to disturb cellular functions by binding to thiol groups of biomolecules. Consequently, a possible therapy for metal intoxication is to remove the toxic metals from the bound functional bioligands by administering strong thiol-containing chelators (Lynn et al., 1999). One of these metal chelators, 2,3-dimercaptopropanol (BAL), has been used in the treatment of poisoning by several heavy metals (Klaassen, 1990). Although, BAL has the capacity to ameliorate the deleterious effects of metal intoxication, it has a low therapeutic index (Andersen, 1989). An important aspect of BAL toxicology is the compound capacity to mobilize the intoxicating metal ion, e.g., arsenic or mercury, to the central nervous system (Aposhian et al., 1996; Emanuelli et al., 1996).

Actually, in animal models of mercury or arsenic intoxication, BAL treatment induces redistribution of these elements from peripheral organs to the brain (Aposhian et al., 1995; Emanuelli et al., 1996). Despite relevant evidence of the BAL therapeutic use, obtained from in vivo and in vitro animal data as well as clinical accounts, its use for metal poisoning treatment has been halted by data suggesting serious neurotoxicity (Pepin et al., 1995; Nogueira et al., 2000, 2001a, b). BAL has been shown to interfere with neurotransmitter systems, including the glutamatergic and GABAergic systems (Nogueira et al., 2000, 2001a, b).

Other metal chelators, such as *meso*-2,3-dimercaptosuccinic acid (DMSA) and 2,3-dimercaptopropane-1-sulfonic acid (DMPS), have also been shown to be effective for treating the toxicity induced by a number of heavy metals (Aposhian et al., 1992; Andersen, 1989;

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Smith et al., 2000). These compounds are more hydrophilic and less toxic than BAL (Aposhian et al., 1995; Domingo, 1995).

Since the therapeutic mechanism underlying the action of these compounds involves promoting metal excretion from the body, potential interaction between clinically employed chelating agents and endogenous metals, for instance, zinc, is probable (Cantilena and Klaassen, 1981). In fact, literature data indicated that DMSA and DMPS increase the urinary output of Cu and Zn in rats (Khandelwal et al., 1987) and in humans (Torres-Alanis et al., 2000; Smith et al., 2000).

Endogenous metals are essential components of many enzyme systems, for instance, δ-aminolevulinate dehydratase (δ-ALA-D) is a metalloenzyme requiring zinc ions for activity (Jaffe et al., 1995). δ-ALA-D catalyses the asymmetric condensation of two molecules of δ-aminolevulinic acid (δ-ALA) to porphobilinogen in the initial steps of heme biosynthesis (Gibson et al., 1955). δ-ALA-D is a sulfhydryl-containing enzyme (Gibson et al., 1955; Barnard et al., 1977), and numerous metals such as mercury (Rocha et al., 1993, 1995), lead (Rodrigues et al., 1989, 1996; Goering, 1993), and other compounds that oxidize sulfhydryl groups modified its activity (Emanuelli et al., 1996; Barbosa et al., 1998; Flora et al., 1998, 2002; Jacques-Silva et al., 2001). Therefore, δ-ALA-D is inhibited by substances that compete with zinc and/or that oxidize the –SH groups (Vieira et al., 2000; Farina et al., 2001, 2002; Bolzan et al., 2002; Nogueira et al., 2003) and is linked to situations associated with oxidative stress (Folmer et al., 2002; Pande et al., 2001; Pande and Flora, 2002; Tandon et al., 2002).

The present study investigated the effects of BAL, DMPS, and DMSA on human blood δ-ALA-D activity in vitro. Of particular importance, this report is based on the following observations: (i) Crude preparation of δ-ALA-D from tissues presents different sensitivity to inhibitors and activators possibly due to endogenous factors specific to each tissue. (ii) The study of blood is important because it is a tissue that can be used to detect possible toxic effects of dithiols. (iii) Previously, our group reported that BAL inhibits δ-ALA-D activity from various tissues in mice (Emanuelli et al., 1998) and that zinc reverses dithiol-induced enzyme inhibition. However, the effects (protecting or restoring) of DTT and Zn^{2+} on the inhibitory action of DMSA and DMPS were not evaluated. These aspects were investigated in detail in the present report. (iv) As pointed out above, tissue factors can interfere with δ-ALA-D and inhibitor interaction. To circumvent this problem we also assessed the effects of DMSA, DMPS, and BAL on purified bovine (Sigma) δ-ALA-D. The roles of zinc chloride ($ZnCl_2$) and reducing protector agents (DTT and cysteine) were also investigated.

2. Materials and methods

2.1. Chemicals

δ-Aminolevulinic acid (δ-ALA), δ-aminolevulinic dehydratase (δ-ALA-D), zinc chloride ($ZnCl_2$), *meso*-2,3-dimercaptosuccinic acid (DMSA), 2,3-dimercaptopropane-1-sulfonic acid (DMPS), and 2,3-dimercaptopropanol (BAL), *p*-dimethylaminobenzaldehyde, and dithiothreitol (DTT) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.2. Samples

Heparinized venous blood was obtained from human volunteers from our work group, University Federal Santa Maria, RS, Brazil.

2.3. Enzyme assays

The activity of blood δ-ALA-D was assayed according to the procedure of Berlin and Schaller (1974). The principle of the method is based on incubation of the enzyme with excess δ-aminolevulinic acid. The porphobilinogen, which is formed within a fixed time, is mixed with modified Ehrlich's reagent, and the color developed is measured photometrically (555 nm) against a blank.

The effects of dithiol chelating agents on erythrocyte δ-ALA-D activity were determined with and without a 10-min preincubation. In the experiments with preincubation, blood enzyme was allowed to react with BAL, DMPS, or DMSA for 10 min before substrate addition or started by addition of blood to the reaction medium containing substrate. Incubations were carried out for 90 min at 37°C.

2.4. Protective effect of thiol-reducing agents or $ZnCl_2$ on δ-ALA-D inhibition induced by chelating agents

To investigate the possible involvement of cysteinyl groups in the inhibitory actions of BAL, DMPS, and DMSA, the protective effects of thiol-reducing agents or $ZnCl_2$ were examined.

The blood samples were preincubated with zinc chloride (0–2 mM) or thiol-reducing agents such as DTT and cysteine (0–3 mM) at 37°C for 10 min. After this time, BAL, DMPS, or DMSA (4–100 μM) was added to the reaction medium, followed immediately by the addition of substrate (δ-ALA).

2.5. Effect of DTT or $ZnCl_2$ as restoring agent for δ-ALA-D inhibition induced by chelating agents

To assess the capacity of DTT or $ZnCl_2$ to reverse δ-ALA-D inhibition caused by DMSA, DMPS, and BAL, they first were preincubated with blood for 10 min at 37°C. After this time, the reaction was started by the addition of substrate (δ-ALA), followed immediately by addition of DTT or $ZnCl_2$.

2.6. Purified δ-ALA-D activity

The purified enzyme (Sigma, 5 units, 2.5 units/mg protein) δ-ALA-D was resuspended in 1 mL medium containing 50 mM potassium phosphate buffer, pH 6.4, 2.5 mM ammonium sulfate, and 0.1 mM dithiothreitol and stored (±4°C) for 7 days. The enzyme (50 μL) was resuspended just before use by incubating with 5 μL of 70 mM DTT and 5 μL of 140 μM $ZnCl_2$ and reactivated at 45°C for 15 min. Enzyme was then diluted 50 times with ultra-purified water.

2.7. Statistical analysis

Results were analyzed by ANOVA, followed by Duncan's multiple-range test when appropriate. Differences between groups were considered significant at $P<0.05$.

3. Results

Human blood δ-ALA-D activity was significantly reduced by dithiol chelating agents in a concentration-dependent manner. BAL and DMSA (1 mM) inhibited significantly enzyme activity (28 and 37%, respectively). DMPS also inhibited blood δ-ALA-D, and its inhibitory effect appears at a concentration (10 μM) lower than that required by BAL and DMSA to inhibit enzyme (Fig. 1).

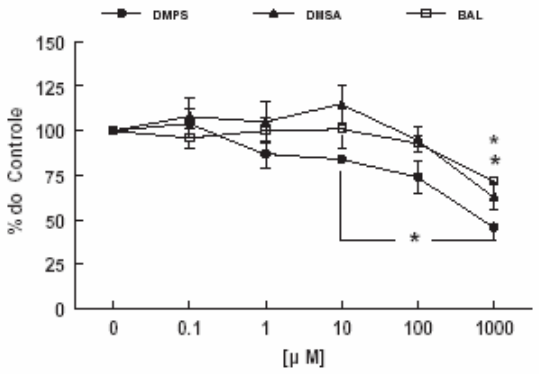


Fig. 1. Inhibitory effect of dithiol chelating agents BAL, DMPS, and DMSA on δ-ALA-D from human blood. δ-ALA-D reaction was started by adding substrate (ALA) to a final concentration of 4.5 mM and incubated for 90 min at 37°C. Data are expressed as means ±SEM for four independent experiments. *Significant difference from control activity $P<0.05$ by Duncan's tests.

3.1. Protective effect of thio-reducing agent or $ZnCl_2$ on δ-ALA-D inhibition induced by chelating agents

Cysteine did not protect δ-ALA-D against the inhibitory effects of DMPS and DMSA (1–4 mM). Conversely, cysteine (3 mM) afforded modest protection against 1 mM BAL inhibition, but did not protect inhibition caused by 4 mM BAL on human erythrocytes δ-ALA-D (Fig. 2A and B).

Dithiotreitol, a compound that protects δ-ALA-D from classical sulfhydryl reagents (Rodrigues et al.,

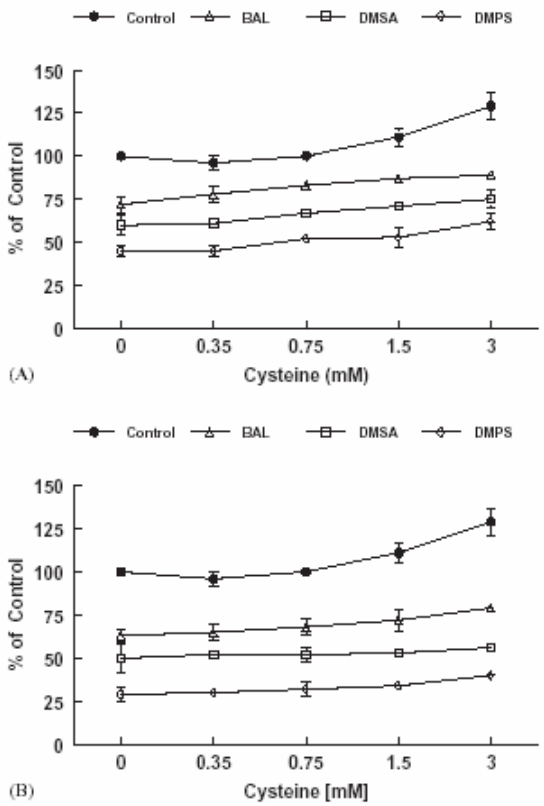


Fig. 2. Effect of cysteine on blood δ-ALA-D inhibition caused by BAL, DMPS, and DMSA 1 mM (2A) and 4 mM (2B). Blood was preincubated with increasing concentrations of cysteine (0–3 mM) at 37°C for 10 min. After 10 min dithiol chelating agents were added, and enzymatic reaction was initiated by adding the substrate (ALA) to a final concentration of 4.5 mM in a medium containing 110 mM phosphate buffer, pH 6.8. Data are expressed as means ±SEM of six independent experiments. *$P<0.05$ compared to control activity (ANOVA/Duncan).

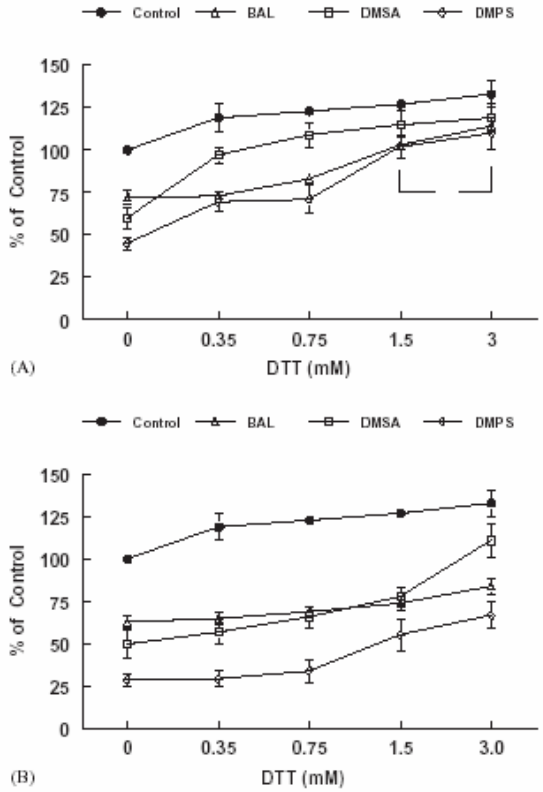


Fig. 3. Effects of dithiothreitol (DTT) on blood δ-ALA-D inhibition caused by BAL, DMPS, and DMSA 1 mM (3A) and 4 mM (3B). Blood was preincubated with increasing concentrations of DTT (0–3 mM). After 10 min dithiol chelating agents were added and enzymatic reaction was initiated by adding the substrate (ALA) to a final concentration of 4.5 mM in a medium containing 110 mM phosphate buffer, pH 6.8. Data are expressed as means ±SEM of five independent experiments. $^{*}P<0.05$ compared to control activity (ANOVA/Duncan).

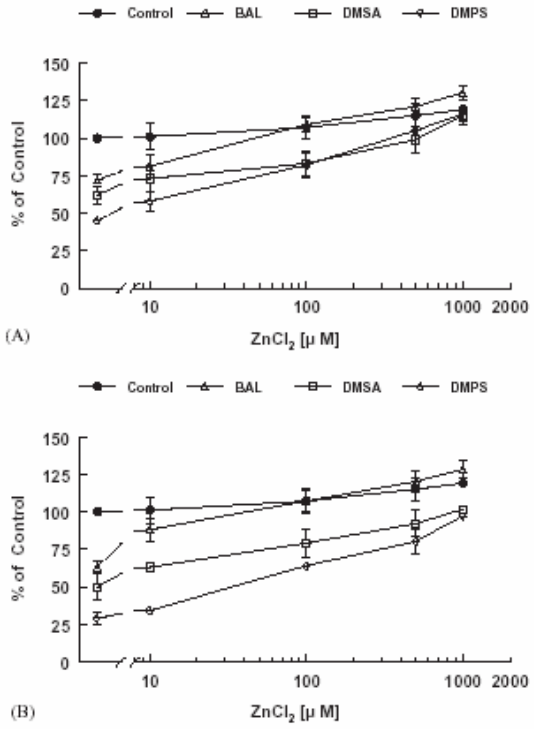


Fig. 4. Effects of BAL, DMPS, and DMSA at 1 mM (4A) and 4 mM (4B) on δ-ALA-D from human blood preincubated with $ZnCl_2$ at different concentrations. The δ-ALA-D reaction was started by adding substrate (ALA) to a final concentration of 4.5 mM and incubated for 90 min at 37°C. Data are expressed as means ±SEM for four independent experiments. $^{*}P<0.05$ compared to control activity (ANOVA/Duncan).

1989), was able to protect the inhibition caused by 1 mM BAL (28%), DMPS (55%), and DMSA (37%) in a concentration-dependent manner (Fig. 3A). However, DTT did not protect the inhibition caused by 4 mM BAL (37%) and DMPS (71%) (Fig. 3B). The protective effect of increasing concentrations of DTT on the inactivation of blood δ-ALA by 4 mM DMSA (50%) is shown in Fig. 3B. At 3 mM, DTT restored the enzyme activity to levels similar to that measured in the absence of inhibitors.

The inhibitory effects of dithiol agents could also be related to its chelating property, which may remove Zn^{2+} essential for δ-ALA-D activity (Tsukamoto et al., 1979, 1980). Zinc chloride up to 100 μM protected 1 and 4 mM BAL inhibitory effect (Fig. 4A and B). Preincubation of enzyme with $ZnCl_2$ up to 500 μM protected against the inhibitory effect caused by 1 mM DMPS and DMSA (Fig. 4A). When DMPS and DMSA (4 mM) were incubated with blood enzyme first preincubated with $ZnCl_2$ (1 mM), no δ-ALA-D inhibition was observed (Fig. 4B).

3.2. Effect of DTT or $ZnCl_2$ as restoring agent for δ-ALA-D inhibition induced by chelating agents

The preincubation of BAL, DMPS, or DMSA (1 or 4 mM) with δ-ALA-D from human erythrocytes increased the inhibitory potency of these compounds (compare Fig. 1 with Fig. 5).

DTT (3 mM) was able to restore the inhibition of δ-ALA-D caused by both 1 mM BAL and DMPS. In contrast, the effect of DMSA (1 mM) was not reversed at control levels by DTT up to 3 mM (Fig. 5A). Equally, DTT did not restore to control levels, inhibition caused by BAL, DMPS, or DMSA (4 mM) on human erythrocytes δ-ALA-D (Fig. 5B).

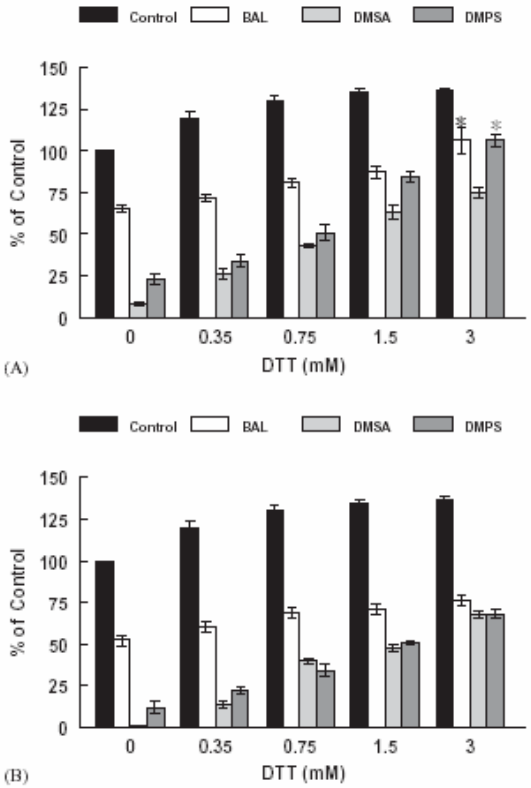


Fig. 5. Effects of dithiothreitol (DTT) on blood δ-ALA-D inhibition caused by BAL, DMPS, and DMSA 1 mM (5A) and 4 mM (5B). Blood was preincubated with 1 or 4 mM dithiol chelating agents. After 10 min, DTT was added, and the enzymatic reaction was initiated by adding the substrate (ALA) to a final concentration of 4.5 mM in a medium containing 110 mM phosphate buffer, pH 6.8. Data are expressed as means ±SEM of five independent experiments. *$P<0.05$ compared to control activity (ANOVA/Duncan).

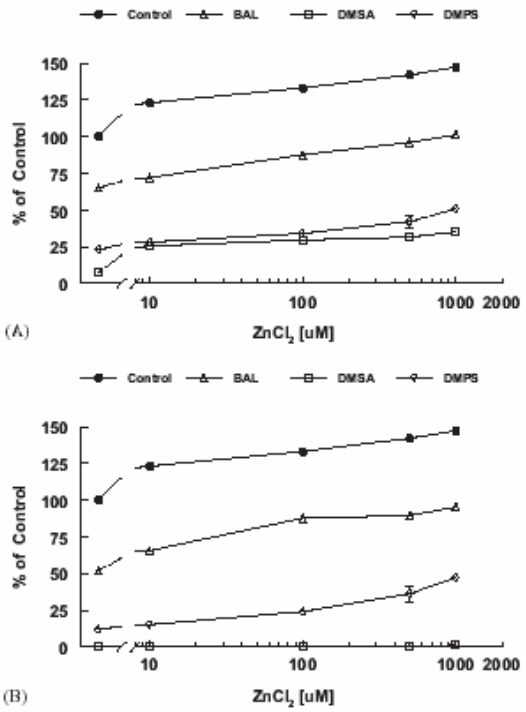


Fig. 6. Effects of BAL, DMPS, and DMSA at 1 mM (6A) and 4 mM (6B) on δ-ALA-D from human blood. Blood was preincubated with dithiol chelating agents. After 10 min, $ZnCl_2$ was added, and the enzymatic reaction was initiated by adding the substrate (ALA) at a final concentration of 4.5 mM in a medium containing 110 mM phosphate buffer, pH 6.8. Data are expressed as means ±SEM of five independent experiments. *$P<0.05$ compared to control activity (ANOVA/Duncan).

$ZnCl_2$ did not reverse the inhibitory effects of DMPS and DMSA (1 and 4 mM) (Fig. 6A and B). However, addition of $ZnCl_2$ (500 μM) after preincubation of BAL (1 mM) in the reaction mixture restored partially the δ-ALA-D activity (Fig. 6A). Similarly, enzyme activity inhibited by the highest concentration of BAL (4 mM) had activity partially restored by adding $ZnCl_2$ (1 mM) (Fig. 6B).

The preincubation of dithiol chelating agents with blood enzyme demonstrated that DMSA was the most potent δ-ALA-D inhibitor from human erythrocytes. These data are in agreement with δ-ALA-D activity from purified enzyme (Sigma) (Table 1). When $ZnCl_2$ (1 μM) was added to the reaction mixture, it increased enzyme activity. Conversely, $ZnCl_2$ (10 μM) increased inhibitory effect of BAL and DMSA. However, DTT (100 μM) totally restored the enzyme activity for all chelating agents tested (Table 2).

Table 1
Purified δ-ALA-D activity in the presence of BAL, DMPS and DMSA at different concentrations

Concentration (μM)	BAL	Compound	
		DMPS	DMSA
0.0	100	100	100
0.5	91.0±0.5	103.0±2.8	95.0±4.6
1.0	82.0±5.7	96.0±2.8	83.0±7.3*
3.0	94.0±5.5	75.0±5.1*	45.7±2.0*
5.0	66.0±4.0*	54.0±4.6*	17.0±1.6*
10.0	52.7±9.0*	43.7±3.9*	14.0±1.4*

Data are expressed as means ±SEM of four independent experiments.
*Significantly different from control (without chelating agents).

4. Discussion

The present investigation clearly indicates that BAL, DMSA, and DMPS inhibit human erythrocyte

δ-ALA-D activity. Of particular importance, the inhibition of δ-ALA-D can lead to accumulation of ALA (Bechara et al., 1993; Emanuelli et al., 2001), which may autoxidize to form reactive oxygen species, such as hydroperoxides (Douki et al., 1998). Reactive oxygen species are toxic, because they may oxidize numerous biomolecules leading to tissue injury and cell death (Yu, 1994).

Metal chelating agents such as EDTA and BAL inhibit δ-ALA-D by removing zinc from the site involved in maintaining cysteinyl residues in a reduced state (Jaffe et al., 1995; Beber et al., 1998; Emanuelli et al., 1998). In contrast with BAL, the DMSA and DMPS inhibitory effects are apparently not related to Zn^{2+} chelation, because Zn^{2+} did not reverse the inhibitory properties of DMSA or DMPS. These results are in agreement with previous data showing that zinc chloride does not change rat liver δ-ALA-D inhibition caused by DMSA and DMPS (Nogueira et al., 2003). Here, we show that if enzyme is first preincubated with high concentrations of $ZnCl_2$, the inhibitory effects of DMSA and DMPS can be reduced. Taken together, these results lead us to propose the following sequence of events to explain the inhibitory effect of DMSA and DMPS: When the enzyme is first preincubated with DMSA and DMPS mixed disulfides between DMPS, DMSA, and δ-ALA-D are formed. The first step in this proposed mechanism involves the removing of Zn^{2+} from δ-ALA-D followed by its oxidation (Scheme 1).

Then, the excess DMSA and DMPS can reduce oxidized δ-ALA-D, forming oxidized DMSA and DMPS. These oxidized compounds form stable mixed disulfides with δ-ALA-D.

The increase in δ-ALA-D sensitivity to dithiol chelating after preincubation indicates that ALA protects the cysteinyl residues at the active site of the enzyme from inactivation by BAL, DMPS, and DMSA. In addition, inhibition of enzyme activity is protected by the mercaptan dithiothreitol, and inhibited δ-ALA-D is restored by subsequent treatment with this compound. Literature data indicate that compounds that oxidized sulfhydryl enzymes can be reversed by dithiols instead of monothiols, suggesting that these inhibitors bind in two vicinal sulfhydryl groups of the enzyme (Laden and Porter, 2001; Gupta and Porter, 2002). Moreover, the greater potency of dithiol (DTT) than of monothiol (cysteine) to restore inhibition caused by chelating agents could be related to sterical factors that allow DTT to reduce the oxidized δ-ALA-D. From these findings, we imply that dithiol chelating agents inactivate human erythrocyte δ-ALA-D by interaction with the vicinal sulfhydryl groups essential for enzyme activity. These groups are likely to be those from the ZnB site in mammalian enzyme. Until recently, the zinc bound to this site was thought to have a structural role in enzyme (Dent et al., 1990; Jaffe et al., 1995; Beber et al., 1998; Emanuelli et al., 1998). However, Jaffe and co-workers, using mutants of human δ-ALA-D, where ZnB or ZnA sites were deleted, demonstrated elegantly that the ZnB site is essential for catalysis, whereas ZnA seems to have no role in enzyme catalysis (Jaffe et al., 2001). Whether Zn^{2+} participates directly in the catalytic cycle or indirectly by maintaining the cysteinyl residues in a reduced state is still unresolved. It is a matter of fact that sulfhydryl groups must be reduced to enzyme show catalytic activity.

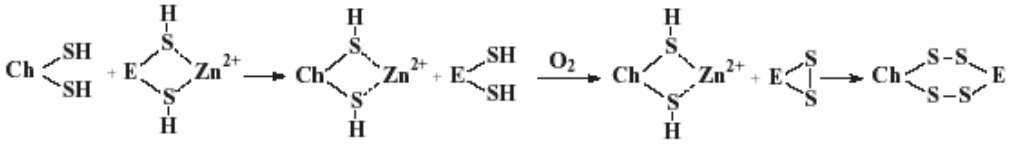
In addition, DTT (−0.33 mV), the thiol with a higher eletronegative redox potential than cysteine (−0.22 mV), reactivated more efficiently with the enzyme inhibited by chelating agents. These results suggest that thiol electronegative redox potential can partially explain the differences on the capacity of cysteine and DTT in restoring chelating agent-inhibited blood δ-ALA-D. Alternatively, either the higher standard redox potential or the sterical factors contribute to the observed high efficiency of DTT when compared to cysteine.

Ercal et al. (1996) pointed out that there is validity in the notion that DMSA may participate in enhancing

Table 2
Purified δ-ALA-D activity preincubated with Zn^{2+} or DTT in the presence of BAL, DMPS, and DMSA

Chelating (μM)		δ-ALA-D activity (%)		
		+ Zn 1μM	+ Zn 10 μM	+ DTT 100μM
Control	100	99.6±4.6	74.3±3.5	100
DMSA 3.0	45.7±2.0*	81.3±5.7*	48.7±2.7*	102.0±3.7
DMPS 10.0	43.7±3.9*	108.7±2.1	70.0±3.7	141.7±4.2*
BAL 10.0	52.7±9.0*	78.0±4.3*	57.0±1.7*	118.7±3.6*

Data are expressed as means ±SEM OF four independent experiments.
*Significantly different from control (without chelating agents).



Scheme 1. Ch, chelating agent (DMSA, DMPS, or BAL); E, enzyme (δ-ALA-D).

oxidative damage to organs. Accordingly, this report showed that DMSA was the most potent δ-ALA-D inhibitor from human erythrocytes and purified enzyme (Sigma). Purified enzyme (Sigma) from bovine liver was significantly inhibited by low dithiol concentrations (Table 1). The higher sensitivity of the purified enzyme to these inhibitors compared to the enzyme from blood could be related to the capacity to form mixed disulfides. In addition, there is evidence suggesting that DMSA chelation therapy could have problematic side effects (Chen et al., 1999).

Another aspect that must be addressed is the possibility that the inhibiting effect of chelators on enzyme activity can be misinterpreted in heavy-metal-intoxicated patients because δ-ALA-D is also inhibited by Pb^{2+} and, to a lesser extent, by other heavy metals. Furthermore, the presence of endogenous substances may also have modified the inhibitory effects of chelators on blood δ-ALA-D. Additionally, literature data have indicated that δ-ALA-D polymorphism may influence an individual's level of lead in blood (Hu et al., 2001; Fleming et al., 1998; Wetmur et al., 1991) and, consequently, the enzyme sensitivity to exogenous factors such as chelating agents.

The results of the present study suggest that it is necessary to investigate the potential toxicity of the chelating agents. Because observations in vitro on chelating toxicity provide only a guide to the toxicity of these compounds, clinical studies are necessary to determine the adverse effects of chelating agents.

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4.1.2 – **Artigo 2**

2,3 DIMERCAPTOPROPANOL, 2, 3-DIMERCAPTOPROPANE-1-SULFONIC ACID AND *MESO*-2, 3-DIMERCAPTOSUCCINIC ACID ACUTE ADMINISTRATION CHANGE DIFFERENTIALLY BIOCHEMICAL PARAMETERS IN MICE

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Short Communication

2,3-Dimercaptopropanol, 2,3-Dimercaptopropane-1-sulfonic Acid and *meso*-2,3-Dimercaptosuccinic Acid Acute Administration Diferentially Change Biochemical Parameters in Mice

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Chelating agents have been used clinically as antidotes for acute and chronic metal intoxications. These compounds not only enhance excretion but, in at least some cases, they also decrease the metal's toxicity by preventing it from binding to cellular target molecules (Aposhian *et al.* 1995). Conversely, prolonged treatment with a chelating agent may lead to haematopoietic disorders (Flora & Kumar 1993), impairment of cellular metabolism, and synthesis of DNA, RNA and protein (Fischer *et al.* 1975), or trace element imbalance (Cantilena & Klaassen 1982).

One of these metal chelators, 2,3-dimercaptopropanol (BAL), has the capacity to ameliorate the deleterious effects of metals intoxications, but it has a low therapeutic index (Andersen 1989). Despite relevant evidence of the BAL therapeutic use, obtained from *in vivo* and *in vitro* animal data as well as clinical accounts, its use as treatment for poisoning has been halted by data suggesting serious neurotoxicity (Pepin *et al.* 1995; Nogueira *et al.* 2000, 2001a&b). Other chelating agents, such as 2,3-dimercapto-1-propanesulfonic acid (DMPS, Dimaval®) and *meso*-2,3-dimercaptosuccinic acid (DMSA, succimer) are less toxic and more effective chelating agents than the chemically analogous BAL (Aposhian *et al.* 1995). DMPS is registered in Germany for the treatment of mercury intoxication (Clarkson *et al.* 1981) or lead poisoning in children and adults (Kemper *et al.* 1990). Clinical reliance on DMSA, an orally administered chelating, has expanded greatly during the last years particularly after its approval for the clinical use against childhood lead poisoning by US Food and Drug Administration (FDA) (Jorgensen 1993).

In the present investigation a number of toxicological parameters on mice acutely treated with BAL, DMPS and DMSA are examined. Of particular importance, we planned this study focus on investigating the activity of a sulfhydryl containing enzyme (δ-ALA-D), since several compounds that oxidize sulfhydryl groups (Emanuelli *et al.* 1996; Flora *et al.* 1998) and compete with zinc (Farina *et al.* 2002; Nogueira *et al.* 2003) inhibit δ-ALA-D activity. TBARS (thiobarbituric acid reactive substances) and tissues zinc concentration were also assessed to study the potential toxicity of chelating.

δ-Aminolevulinic acid (δ-ALA), *meso*-2,3-dimercaptosuccinic acid (DMSA), 2,3-dimercaptopropane-1-sulfonate (DMPS) and 2,3-dimercaptopropanol (BAL), *p*-dimethylaminobenzaldehyde and dithiothreitol (DTT) were purchased from SIGMA (St. Louis, MO, U.S.A). All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Male adult Swiss albino mice (25–35 g) from our own breeding colony were used. The animals were kept on separate animal rooms, on a 12 hr light/dark cycle, at a room temperature of 22°, with free access to food and water.

The animals were divided into four groups (six mice/group): (1) control animals (1 ml/kg, dimethylsulfoxide) or treated groups (2, 3 and 4). Treated mice were administered subcutaneously a single dose (1.6 mmol/kg) of chelating agents as following: (2) BAL, (3) DMPS and (4) DMSA. After 24 hr, DMPS or DMSA treated animals were sacrificed under light ether anaesthesia and blood was collected by cardiac puncture in heparinized tubes. Brain, liver and kidney were also removed. The animals treated with BAL, which at this dose induces tonic-clonic seizures (Nogueira *et al.* 2000), were killed afterwards (15 min.) the convulsive episode. DMPS- and DMSA-treated mice presented no tonic-clonic seizures nor apparent clinical symptoms.

The activity of blood δ-ALA-D was assayed according to the method of Berlin & Schaller (1974) and tissues δ-ALA-

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Table 1.

Effect of acute treatment with chelating agents on δ-ALA-D activity in mice blood, liver, kidney and brain.

Group	Blood	Liver	Kidney	Brain
Control	100±6	100±6	100±6	100±6
BAL	130±14	117±16*	73±6*	136±26*
DMPS	162±13	97±8	64±16*	89±28
DMSA	112±11	104±5	97.5±16.55	80±27

The enzyme activity of blood, liver, kidney and brain for control group was 3.02±0.017, 14.35±1.0, 7.3±0.07, 5.23±0.03 nmol PBG/ mg protein/ hour, respectively. Data are expressed as mean±S.D. of six animals per group. (*) Denoted P<0.05 as compared with control group (ANOVA/Duncan).

D activity were assayed according to the method of Sassa (1982) by measuring the rate of product (porphobilinogen) formation. Tissues TBARS was determined as described by Ohkawa *et al.* (1979). Brain, liver and kidney metal concentration was determined by graphite furnace atomic absorption spectrometry using a Varian SpectrAA 200 spectrometer (Melbourne, Australia) (Welz & Sperling 1999). Protein was measured by the Coomassie blue method according to Bradford (1976) using bovine serum albumin as standard. Data are expressed as means±S.D. Statistical analysis was performed using a one-way ANOVA followed by the Duncan's test. Values of P<0.05 were considered statistically significant.

Acute DMPS exposure significantly enhanced blood δ-ALA-D activity and enzyme activity reached 162% of control group (P<0.05). However, there was no alteration in blood enzyme activity in BAL- or DMSA-treated mice (table 1). No change in δ-ALA-D activity of hepatic tissue in DMPS- or DMSA-treated mice was observed. However, BAL-treated mice presented increase on hepatic enzyme activity (F (3,18)=4.35; P<0.05) (table 1). A significant decrease in renal δ-ALA-D activity was observed in BAL- and DMPS-treated mice. In fact, one-way ANOVA revealed a significant effect for kidney enzyme (F (3,22)=13.93; Ps<0.05). Conversely, DMSA-treated mice did not alter the activity of kidney enzyme (table 1). No change on δ-ALA-D activity from brain tissue in DMPS- or DMSA-treated mice was observed. Animals that received a single dose of BAL presented increase on brain enzyme activity (F (3,24)= 8.28; P<0.05) (table 1).

The effect of chelating administration on zinc concentration in liver, brain and kidney is presented in table 2. DMPS-treated mice showed an increase in renal zinc concentration (F (3,10)=7.20; P<0.05). However, DMPS did not alter liver and brain zinc concentrations when compared to the control group. Decrease in hepatic zinc concentration occurred in mice acutely administered BAL (F (3,18)=3.49; P<0.05), while no significant effect was observed in brain and kidney. Acute administration of DMSA did not influence zinc concentration in all tissues evaluated (table 2).

A significant increase in liver (F(3,20)=5.38; P<0.05) and kidney (F(3,20)=8.59; P<0.05) lipid peroxidation was induced by administering BAL, DMPS or DMSA in mice. There was no significant effect of chelating agents on lipid peroxidation to mice brain (table 2).

The main purpose of this study was to identify the toxic effects of BAL, DMSA and DMPS using an acute intoxication model in mice. Our experimental rationale was based on neurotoxic effects induced by the particular dose of BAL used in this study. Our first finding is that BAL and DMPS are toxic when administrated acutely, considering δ-ALA-D inhibitory effect (table 1) and increase of TBARS levels on mice kidney (table 2). Hence, δ-ALA-D plays a fundamental role in most aerobic organisms by participating in haem, biosynthesis an inhibition on δ-ALA-D activity can impair haem biosynthesis and contribute to increase ALA accumulation, which in turn can enhance generation of free radicals, aggravating oxidative damage to cell components (Bechara 1996). Consequently, we presume that δ-ALA-D inhibition participates, at least in part, in the acute toxicity manifestations caused by BAL and DMPS. In line with this, recent evidence from our and other laboratories have demonstrated that reduction of δ-ALA-D of the same magnitude as that observed here is associated with an increase in oxidative stress in rodents (Flora *et al.* 2002; Soares *et al.* 2003).

Besides, a single dose of DMPS increased 1.7 times the Zn level of kidney. The increase of Zn tissue level after administration of DMPS may be associated with mobilization of this metal from other organs to kidney. Since the thera-

Table 2.

Effects of chelating exposure on zinc concentration and lipid peroxidation in mice liver, kidney and brain.

	Zinc concentration (% of control)			MDA levels (% of control)		
Group	Liver	Kidney	Brain	Liver	Kidney	Brain
Control	98.0±5	101.0±6	99.0±4	105.0±10	103.6±9	106.5±11
BAL	76±22*	110.0±9	109.0±14	137.5±15*	153.0±28*	93.0±9
DMPS	106±17	175.0±48*	112.0±10	138.0±31*	146.0±24*	102.0±16
DMSA	104±22	105.0±13	117.0±19	139.0±14*	157.0±21*	89.0±10

Zinc concentration is expressed as percentage of control group. Zinc concentration (μg/g) was 16±1.8 (compared to control group). The MDA control levels were 84.75±36.47 (brain), 69.63±9.23 (liver) and 390.63±46.24 nmol MDA/ g tissue. Control animals received dimethylsulfoxide (1 ml/kg). Data are reported as mean± S.D. of 4–6 animals per group. (*) Denoted P<0.05 as compared to control group (ANOVA/Duncan).

peutic mechanism underlying the action of these compounds involves promoting metal excretion from the body, potential interaction between clinically employed chelating agents and endogenous metals, for instance zinc, is probable (Cantilena & Klaassen 1981).

The concentrations of zinc in most tissues of several mammalian species studied are in the order of 10 to 100 μg/g wet weight, with little variation among species (Simon & Taylor 2001). Since the body can tolerate ranges in tissue levels of essential elements, we can infer that this slight significant increase in kidney zinc level (28 μg/g, Table 1) was possibly not by itself the mediator of DMPS effects. Thus, theDMSA-treated mice had no change in either Zn levels or δ-ALA-D activity in any tissue studied. Likewise, DMSA treatment had no influence on the Zn levels of blood, brain or kidney in rats (Tandon *et al.* 2002).

The second point of interest is that BAL inhibited δ-ALA-D activity not by removing zinc from the kidney and the enzyme in this intoxication protocol. In fact, BAL did not change kidney zinc concentration but inhibited renal enzyme. Conversely, hepatic enzyme activity was stimulated and zinc liver concentration was significantly reduced (12 μg/g) in BAL-treated mice. It is important to consider that even though zinc concentration was significantly different from control group, this value is still on the tolerated range of zinc in hepatic tissue. This result may be of considerable importance because literature data have shown that EDTA and BAL inhibit δ-ALA-D *in vitro* by removing zinc from the site involved in maintaining cysteinyl residues in a reduced state (Emanuelli *et al.* 1998). In addition, our *in vivo* results are in accordance with a previous study where BAL also inhibited renal δ-ALA-D (Emanuelli *et al.* 1996) in mice.

Third, the effects of these chelators on δ-ALA-D activity varied among tissues (increases in blood, liver and brain versus decreases in kidney), and varied among experiments (*in vivo* and *in vitro*). Furthermore, the differences among chelators are also related to their intrinsic chemical and physical properties. In fact, BAL is more hydrophobic than DMPS and DMSA and has greater ability to enter into the brain than the more hydrophilic analogues.

DMPS and DMSA did not alter hepatic δ-ALA-D activity (table 1) in this study, as opposed to a concentration-dependent inhibition of hepatic δ-ALA-D *in vitro* (Nogueira *et al.* 2003). Recently we demonstrated that BAL, DMPS and DMSA inhibit human blood δ-ALA-D activity, *in vitro* (Nogueira *et al.* 2004). In contrast, chelating agents acutely administered in mice did not inhibit blood δ-ALA-D activity. A possible explanation for these controversies is that high concentrations of chelators can not be reached in tissues *in vivo*. As well, when experiments were performed *in vitro*, chelating agents were allowed to react with enzyme before being added to ALA substrate. When the enzyme is first preincubated with chelating stable mixed disulfides are formed, consequently the enzyme is inhibited (Nogueira *et al.* 2004).

The generation of free radicals aggravates oxidative damage to cell components and these events can contribute to promoting chelating toxicity in our treatment. However it is not known whether some of the toxic side-effects associated with the use of the chelating agents are related to oxidative stress (Mehta & Flora 2001).

In this way, the results reported here clearly indicate that DMSA produced fewer biochemical changes than the other two chelators tested. However, the exact mechanism the chelating toxicity is still unclear but can involve a variety of molecular or cellular targets.

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4.2 – Possível papel protetor dos agentes quelantes (DMPS e DMSA) e do disseleneto de difenila, $(PhSe)_2$, em modelos de intoxicação por cádmio administrado agudo e sub-crônico em camundongos

4.2.1 – **Artigo 3**

CADMIUM INDUCED TESTICULAR DAMAGE AND ITS RESPONSE TO ADMINISTRATION OF SUCCIMER AND DIPHENYL DISELENIDE IN MICE

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Cadmium induced testicular damage and its response to administration of succimer and diphenyl diselenide in mice

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Abstract

Acute effects of cadmium in mice testes were evaluated. Animals received a single dose of $CdCl_2$ (2.5 mg/kg or 5 mg/kg, intraperitoneally) and a number of toxicological parameters in mice testes were examined such as δ-aminolevulinic acid dehydratase (δ-ALA-D) activity, lipid peroxidation, hemoglobin content and components of the antioxidant defenses (superoxide dismutase (SOD) activity and ascorbic acid concentration). Furthermore, a possible protective effect of meso-2,3-dimercaptosuccinic acid (DMSA) and diphenyl diselenide $(PhSe)_2$ are studied. The results demonstrated inhibition of δ-ALA-D and SOD activities, reduction in ascorbic acid, increase of lipid peroxidation induced by cadmium, indicating testes damage. DMSA (400 μmol/Kg) and $(PhSe)_2$ (100 μmol/Kg) protected inhibitory effect of 2.5 mg/kg $CdCl_2$ on δ-ALA-D and restored the increase of TBARS levels. Otherwise, $(PhSe)_2$ treatment was effective in reducing the increase of TBARS levels induced by 5 mg/kg $CdCl_2$, whereas DMSA and $(PhSe)_2$, in combination, were ineffective in reducing TBARS level. However, these compounds alone or in combination, were unable to protect SOD activity and to improve ascorbic acid levels near to the normal value. The use of combined therapy (DMSA plus $(PhSe)_2$) not proved be better than the monotherapy, in improving toxicological parameters evaluated in this model of testicular damage induced by cadmium.
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Keywords: Antioxidant; Cadmium; DMSA; Selenium; Organoselenium; Testes

1. Introduction

Chelation therapy is the most effective means of treating metal intoxication. The prognosis in acute and chronic human intoxication for a range of metals can be improved considerably by administration of suitable chelating agent (Mehta and Flora, 2001). These compounds bind to and enhance the excretion of toxic elements such as arsenic, cadmium, lead, or mercury. Moreover, chelators not only enhance excretion but, in some cases, they also decrease the metal's toxicity by preventing it from binding to cellular target molecules (Domingo, 1995). However, prolonged treatment with a chelating agent may lead to haematopoietic

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disorders (Flora and Kumar, 1993), impairment of cellular metabolism, and synthesis of DNA, RNA and protein (Fischer et al., 1975), or trace element imbalance (Cantilena and Klaasen, 1982).

Clinical reliance on DMSA, an orally administered chelator, has expanded greatly during the last few years particularly after its approval for the clinical use for childhood lead poisoning by US Food and Drug Administration (FDA) (Jorgensen, 1993). Treatment with DMSA has been found to be effective in terms of survival and lowering the cadmium content of liver and kidney in cadmium-exposed mice (Basinger et al., 1988). Moreover, this compound is one of the less toxic drugs that can be given orally a less obvious benefit may also be derived as a result of DMSA's structural potential to serve as an antioxidant in vivo (Ercal et al., 1996).

Of all the toxic metals found in the environment and used in industry, cadmium occupies a special place because of the generally intractable nature of cadmium intoxication (Jones and Cherian, 1990). Acute Cd poisoning produces primarily hepatic and testicular injury, whereas chronic exposure results in renal damage and osteotoxicity (Rikans and Yamano, 2000; Folmer et al., 2004). Several lines of evidence indicate that reactive oxygen species are involved in cadmium-mediated tissue damage. Testes can be particularly affected; a single carcinogenic dose of cadmium can cause significant testes pathology (hemorrhages, atrophy, and calcification) which has been attributed in part to oxidative stress as assessed by an increased lipid oxidation, a decreased in reduced glutathione and an increased H_2O_2 production (Koisumi and Li, 1992). Thus, it is believed that antioxidant should be one of the important components of an effective treatment of cadmium poisoning. In line with this, combined administration of *n*-acetylcysteine (NAC) and succimer caused a rapid mobilization of arsenic and lead (Flora, 1999; Pande et al., 2001), indicating that the effects of combined therapy with antioxidants and chelators can yield better therapeutic outcomes than isolated chelation therapy.

The concept that selenium-containing molecules may be better nucleophiles (and therefore antioxidants) than classical antioxidants, has led to the design of synthetic organoselenium compounds (Arteel and Sies, 2001). Several reports have been published on glutathione peroxidase (GSH-px)-mimetic seleno-compounds, which, like the native enzyme, rely on the redox cycling of selenium. In fact, recent study has shown that the diaryl diselenides were potent antioxidants in mice. Moreover, $(p\text{-ClPhSe})_2$ and $(PhSe)_2$ presented higher thiol peroxidase activity and demonstrated better antioxidant potential than the other diselenides tested (Meotti et al., 2004). A variety of seleno organic compounds are now considered as potential antioxidant and chemopreventive pharmacological agents (Commandeur et al., 2001; Klotz et al., 2003).

Of particular importance, the simplest of diaryl diselenides, diphenyl diselenide $[(PhSe)_2]$ has been shown to be even more active as a glutathione peroxidase mimic (Wilson et al., 1989) and less toxic to rodents than ebselen (Nogueira et al., 2003b; Meotti et al., 2003).

Given the observations described above, in the present work we investigated the beneficial effects of DMSA when given alone or in combination with diphenyl diselenide $(PhSe)_2$ on Cd-induced testicular damage. Thereby, we evaluated the effect of Cd on δ-ALA-D activity, lipid peroxidation, hemoglobin content and components of the antioxidant defenses (SOD and ascorbic acid) in mice testes.

2. Materials and methods

2.1. Chemicals

DMSA (meso-2,3-dimercaptosuccinic acid), $CdCl_2$, δ-aminolevulinic acid (δ-ALA) and *p*-dimethylaminobenzaldehyde were purchased from Sigma (St. Louis, MO, USA). Diphenyl diselenide $(PhSe)_2$ was synthesized according to Paulmier (1986). All other chemicals were of analytical grade and obtained from standard commercial suppliers. DMSA and $(PhSe)_2$ were dissolved in dimethylsulfoxide (DMSO).

2.2. Animals

Male adult Swiss albino mice (25–35 g) from our own breeding colony were used. The animals were kept on separate animal rooms, on a 12 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, School of Medicine Veterinary and Animal Science of the University of Sao Paulo, Brazil.

2.3. Exposure

A group of six to eight mice was usually tested in each experiment. The mice were injected intraperitoneally with a single dose of $CdCl_2$ (2.5 mg/kg or 5 mg/kg) (dissolved in saline at 0.5 mg/ml) and 30 min later they were injected intraperitoneally with 400 μmol/kg DMSA (Xie et al., 1995) or subcutaneouslly with diphenyl diselenide (100 μmol/kg), a effective and non-toxic dose (Nogueira et al., 2003c). The treatment protocol has been choose based on Min et al. (2002), Srivastava et al. (1991) and Oteiza et al. (1999) in attempt to cause a severe testicular injury. The testicular damage was evidenced by increase of hemoglobin content and TBARS levels as described by Koisumi and Li (1992). We also observed hemorrhages of testes in mice treated with both doses of cadmium.

Animals were sacrificed by decapitation 24 h after $CdCl_2$ treatment and then, testes were removed. The protocol of mice treatment is given below:

Group 1—saline (i.p.) + DMSO (s.c.) + DMSO (i.p.).
Group 2—$CdCl_2$ (2.5 mg/kg, i.p.) + DMSO (s.c.) + DMSO (i.p.).
Group 3—$CdCl_2$ (5 mg/kg, i.p.) + DMSO (s.c.) + DMSO (i.p.).
Group 4—saline (i.p.) + DMSO (s.c.) + DMSA (400 μmol/kg, i.p.).
Group 5—saline (i.p.) + $(PhSe)_2$ (100 μmol/kg, s.c.) + DMSO (i.p.).
Group 6—saline (i.p.) + DMSA (400 μmol/kg, i.p.) + $(PhSe)_2$ (100 μmol/kg, s.c.).
Group 7—$CdCl_2$ (2.5 mg/kg, i.p.) + DMSO (s.c.) + DMSA (400 μmol/kg, i.p.).
Group 8—$CdCl_2$ (2.5 mg/kg, i.p.) + $(PhSe)_2$ (100 μmol/kg, s.c.) + DMSO (i.p.).
Group 9—$CdCl_2$ (2.5 mg/kg, i.p.) + DMSA (400 μmol/kg, i.p.) + $(PhSe)_2$ (100 μmol/kg, s.c.).
Group 10—$CdCl_2$ (5 mg/kg, i.p.) + DMSO (s.c.) + DMSA (400 μmol/kg, i.p.).
Group 11—$CdCl_2$ (5 mg/kg, i.p.) + $(PhSe)_2$ (100 μmol/kg, s.c.) + DMSO (i.p.).
Group 12—$CdCl_2$ (5 mg/kg, i.p.) + DMSA (400 μmol/kg, i.p.) + $(PhSe)_2$ (100 μmol/kg, s.c.).

2.4. δ-Aminolevulinic acid dehydratase (ALA-D) activity

Testicular δ-ALA-D activity was assayed according to the method of Sassa (1982) by measuring the rate of product (porphobilinogen) formation except that 45 mM sodium phosphate buffer and 2.2 mM ALA were used. Samples were homogenized in 0.9% NaCl in the proportion (w/v) 1/5 and centrifuged at 2400 × *g* for 15 min. An aliquot of 50 μL of homogenized tissue was incubated for 2 h at 37 °C. Reaction was linear in relation to protein and time of incubation. The reaction product was determined using modified Erlich's reagent at 555 nm.

2.5. Lipid peroxidation

Testes were rapidly homogenized in 50 mM Tris–HCl, pH 7.5 (1/10 (w/v)) and centrifuged at 2400 × *g* for 15 min. An aliquot (200 μL) of homogenized was incubated at 95 °C for 2 h. TBARS was determined as described by Ohkawa et al. (1979).

2.6. Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity in testes was assayed spectrophotometrically as described by Misra and Fridovich (1972). This method is based on the capacity of SOD to inhibit autoxidation of adrenaline to adrenochrome. The color reaction was measured at 480 nm. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinefrine autoxidation by 50% at 26 °C.

2.7. Ascorbic acid determination

Ascorbic acid determination was performed as described by Jacques-Silva et al. (2001). Proteins (testes) were precipitated in 10 volumes of a cold 4% trichloroacetic acid solution. An aliquot of the sample in a final volume of 1 ml of the solution was incubated for 3 h at 38 °C then 1 ml H_2SO_4 65% (v/v)

was added to the medium. The reaction product was determined using color reagent contained 4.5 mg/ml dinitrophenyl hydrazine and $CuSO_4$ (0.075 mg/ml).

2.8. Hemoglobin content and protein determination

Hemoglobin concentration in testes supernatants was assayed using a comercial kit according to the method of Henry et al. (1974) (LABTEST Diagnóstica S.A., Minas Gerais, Brazil). Protein was measured by the Coomassie blue method according to Bradford (1976) using bovine serum albumin as standard.

2.9. Metal determination

Concentration of cadmium in testes was determined by graphite furnace atomic absorption spectrometry using a Varian SpectrAA 200 spectrometer (Melbourne, Australia). The samples were digested with a mixture of HNO_3 and $HClO_4$ concentration 3 + 1 (5 ml) and diluted to 20 ml before measuring their cadmium content (Welz and Sperling, 1999).

2.10. Statistical analysis

Data are expressed as means ± S.E.M. Statistical analysis was performed using a three-way analysis of variance (ANOVA), followed by the 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. For cadmium determination, a non-parametric Kruskal–Wallis test, followed by Mann–Whitney U test was applied because control group had no variance (all values were bellow the detection limit).

3. Results

3.1. δ-ALA-D activity

Three-way ANOVA yielded a significant DMSA × $(PhSe)_2$ × Cd^{2+} interaction ($P < 0.05$). Post-hoc comparisons demonstrated that acute cadmium exposure, at two doses tested (2.5 and 5 mg/kg), significantly inhibited δ-ALA-D activity (65% and 49%, respectively) in testes ($P < 0.0001$). $(PhSe)_2$ and DMSA individually or combined ($(PhSe)_2$ plus DMSA), restored inhibition caused by cadmium on δ-ALA-D activity (Fig. 1). There was no alteration in testes enzyme activity from animals that received DMSA and $(PhSe)_2$.

3.2. Lipid peroxidation

Three-way ANOVA of TBARS levels yield a significant DMSA × $(PhSe)_2$ × Cd^{2+} interaction. Post-hoc comparisons demonstrated that 2.5 and 5 mg/kg Cd^{2+} increased ($P < 0.0001$) lipid peroxidation in testes (1.98- and 3.0-fold higher, respectively, than the control value) and $(PhSe)_2$ treatment was effective in reducing cadmium-induced TBARS increase in testes. DMSA administration was effective in limiting TBARS enhancement caused by 2.5 mg/kg cadmium. On the other hand, this compound was ineffective in restoring TBARS status towards the control level when mice received 5 mg/kg cadmium.

Animals co-administered with $(PhSe)_2$ and DMSA plus 5 mg/kg cadmium, exhibited TBARS levels higher than all the other groups ($P < 0.0001$), while

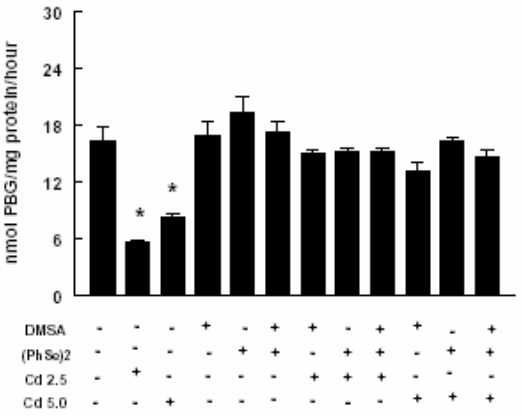

Fig. 1. Effect of $(PhSe)_2$, DMSA or their combination on cadmium-induced alterations in δ-ALA-D activity in testes of cadmium-exposed mice. Tissues were pre-incubated at 37 °C for 10 min. Enzymatic reaction was initiated by adding the substrate (ALA) to a final concentration of 2.2 mM in a medium containing 45 mM phosphate buffer, pH 6.8. Data are expressed as mean ± S.E.M. of seven animals per group. (*) Denoted $P < 0.05$ as compared to control group (three-way ANOVA/Duncan).

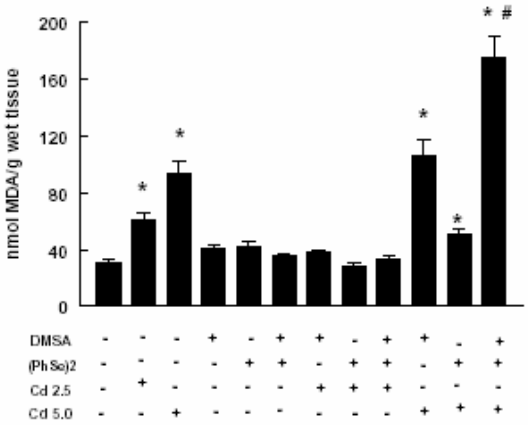

Fig. 2. Effect of $(PhSe)_2$, DMSA or their combination on cadmium-induced alterations in TBARS levels in testes of cadmium-exposed mice. Data are reported as mean ± S.E.M. of seven animals per group. (*) Denoted $P < 0.05$ as compared to control group (three-way ANOVA/Duncan). (#) Denoted $P < 0.05$ as compared to all other experimental groups.

the concomitant therapy was effective in ameliorated TBARS levels when the cadmium dose was 2.5 mg/kg (Fig. 2).

3.3. Ascorbic acid determination

Three-way ANOVA of ascorbic acid content yielded a significant main effect of Cd^{2+} ($P < 0.0001$). Post-hoc comparison demonstrated that acute cadmium exposure, at two doses tested (2.5 and 5 mg/kg), significantly reduced ascorbic acid levels (72% and 65%, respectively) in testes ($P < 0.0001$). Decreased ascorbic acid levels remained unchanged after treatment with DMSA and $(PhSe)_2$, individually or after combined treatment (Fig. 3).

3.4. Hemoglobin concentration

Three-way ANOVA of testicular hemoglobin yielded a significant main effect of Cd^{2+} ($P < 0.0001$). Post-hoc comparisons indicated that cadmium treatment was associated with a significant accumulation of hemoglobin in the testes ($P < 0.0001$), which was 2.4-times (2.5 mg/kg Cd^{2+} group) and 3.2-times (5 mg/kg Cd^{2+} group) higher than in the control group (Fig. 4). $(PhSe)_2$, DMSA or their combination were unable to restore testes hemoglobin content to the normal level.

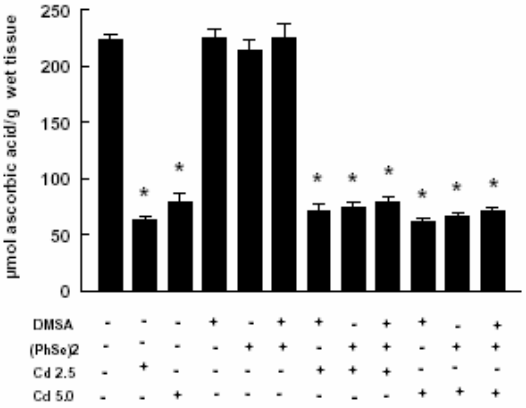

Fig. 3. Effect of $(PhSe)_2$, DMSA or their combination on cadmium-induced alterations in ascorbic acid levels in testes of cadmium-exposed mice. Data are reported as mean ± S.E.M. of seven animals per group. (*) Denoted $P < 0.05$ as compared to control group (three-way ANOVA/Duncan).

3.5. Superoxide dismutase (SOD) activity

Three way ANOVA of SOD revealed a significant main effect of Cd^{2+} ($P < 0.0001$). Post-hoc comparisons demonstrated that 5 mg/kg Cd^{2+} intoxication

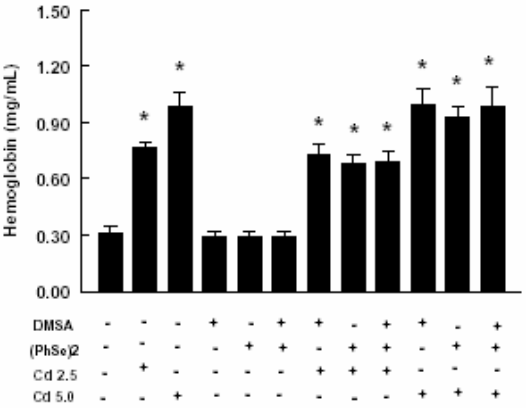

Fig. 4. Effect of $(PhSe)_2$, DMSA or their combination on cadmium-induced alterations in hemoglobin concentration in testes of cadmium-exposed mice. Data are reported as mean ± S.E.M. of seven animals per group. (*) Denoted $P < 0.05$ as compared to control group (three-way ANOVA/Duncan).

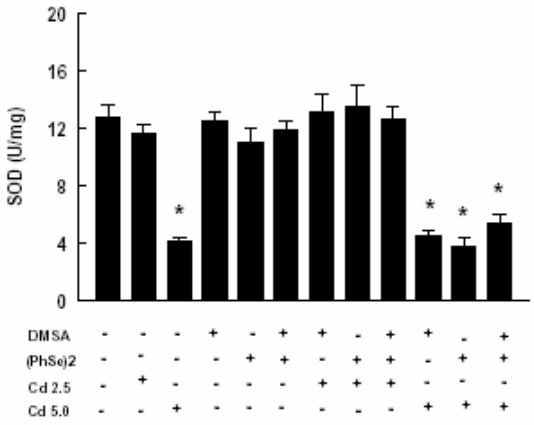

Fig. 5. Effect of $(PhSe)_2$, DMSA or their combination on cadmium-induced alterations in superoxide dismutase activity in testes of cadmium-exposed mice. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinefrine autoxidation by 50% at 26 °C. Data are reported as mean ± S.E.M. of seven animals per group. (*) Denoted $P < 0.05$ as compared to control group (three-way ANOVA/Duncan).

caused a decrease of about three times on SOD activity ($P < 0.0001$); whereas mice exposed to 2.5 mg/kg cadmium did not present change on SOD activity. Inhibited SOD activity remained unchanged on mice treated with DMSA and $(PhSe)_2$, individually or after combined treatment (Fig. 5).

3.6. Cadmtum determtnatton

Testes cadmium accumulation following the doses of 2.5 and 5.0 mg Cd/kg was higher (200- and 320-fold, respectively) than in control mice (Table 1). Furthermore, mice exposed to 5 mg/kg accumulated significantly more cadmium in testes than mice exposed to 2.5 mg/kg. Cadmium levels remained unchanged after treatment with DMSA and $(PhSe)_2$, whereas these compounds, individually or after combined treatment, were inefficient in restoring cadmium levels towards the control level (Table 1).

Table 1
Cadmium concentration in testes of mice exposed to 2.5 and 5 mg/kg cadmium

Groups	Control	Cd (2.5 mg/kg)	Cd (5.0 mg/kg)
Control	<0.2	40 ± 9[a]	64 ± 12[a,b]
DMSA	<0.2	37 ± 2[a]	58 ± 6[a,b]
$(PhSe)_2$	<0.2	30 ± 9[a]	57 ± 9[a,b]
DMSA × $(PhSe)_2$	<0.2	37 ± 10[a]	78 ± 26[a,b]

Data are expressed as μg Cd/g. Data are mean ± S.D. from six animals in each group.

[a] Denoted $P < 0.0001$ as compared to control group by Kruskal–Wallis, followed by Mann–Whitney U test.

[b] Denoted $P < 0.05$ as compared to Cd (2.5 mg/kg) group by Kruskal–Wallis, followed by Mann–Whitney U test (<0.2 was the limit of detection for cadmium determination).

4. Discussion

Acute administration of cadmium caused a marked increase in TBARS production, hemoglobin and a decrease on δ-ALA-D and SOD activities and ascorbic acid content in testes of mice. Cadmium-treated groups demonstrated higher hemoglobin content, which is in agreement with the visual observation of testes hemorrhagic infiltration in these groups. Either monotherapy (DMSA and $(PhSe)_2$) or the combined therapy with these compounds was inefficient in ameliorating the hemorrhage caused by Cd^{2+}.

The toxic action of cadmium is far from being completely understood, although lipid peroxidation has long been considered to be the primary process responsible for cadmium toxicity (Pal et al., 1993; Manca et al., 1991). Really, cadmium induced oxidative damage has been demonstrated by the increase of lipid peroxidation and inhibition of enzymes required to prevent such oxidative damage (Kelley et al., 1999). Accordingly, the present study demonstrated increase of TBARS levels after cadmium-treatment. $(PhSe)_2$ treatment was effective in improving TBARS status towards the normal level. Our group has demonstrated that $(PhSe)_2$ is a potent antioxidant in vitro (Rossato et al., 2002) and presents higher thiol peroxidase activity than other diselenides and ebselen (Wilson et al., 1989, Meotti et al., 2004). Likewise, DMSA was effective in restoring the increase of TBARS levels in testes of mice intoxicated with 2.5 mg/kg cadmium towards the control level. When TBARS production is measured in testes of mice intoxicated with 5 mg/kg cadmium a different effect was observed, since DMSA either alone or in combination with $(PhSe)_2$, was ineffective in reducing TBARS levels. In this way, Pande and Flora (2002) have demonstrated that treatment with DMSA led to a more pronounced increase in TBARS of rat renal tissue compared with lead exposed

animals. Even though, Ercal et al. (1996) have described DMSA as a potential antioxidant in vivo, in our hands this chelating agent fails in restoring TBARS status to control levels. It is interesting to note that at dose of 5 mg/kg cadmium combined therapy, $(PhSe)_2$ plus DMSA, increased TBARS levels when compared with all other groups.

In this work, for the first time, we demonstrated changes in testicular δ-ALA-D activity after cadmium exposure and, for this ending point of toxicity, $(PhSe)_2$ and DMSA were effective in restoring δ-ALA-D activity to control levels. In addition, combined administration of $(PhSe)_2$ with DMSA demonstrated to be similar to the monotherapy with these compounds, in improving testes δ-ALA-D activity towards the normal level. Tentatively, cadmium-induced inhibitory effect could be related with lipid peroxidation since we have described that δ-ALA-D activity is extremely sensitive to situations associated with oxidative stress (Folmer et al., 2002, Pande and Flora, 2002, Soares et al., 2003, Nogueira et al., 2004). Furthermore, mammalian δ-ALA-D is a metalloenzyme that requires Zn^{2+} for maximal catalytic activity and data support the hypothesis of a direct competition between bivalent metals and Zn^{2+} on δ-ALA-D from mammals and bacteria (Tsukamoto et al., 1979; Sommer and Beyersmann, 1984; Rocha et al., 1995; Jaffe, 2000; Nogueira et al., 2003a,b). Thus, Cd^{2+} could cause a Zn^{2+} displacement leading to δ-ALA-D inhibition. However, the exact mechanism to explain the cadmium toxicity is unclear, since both DMSA and $(PhSe)_2$ were effective in restoring δ-ALA-D activity, whereas only $(PhSe)_2$ was effective in ameliorate TBARS levels. DMSA possibly is chelating Cd^{2+}, when the dose studied was of 5 mg/kg, since it was able in restoring enzyme activity without modifying TBARS production. Conversely, at cadmium dose of 2.5 mg/kg, both DMSA and combined therapy (DMSA plus $(PhSe)_2$) were effective in restoring TBARS levels. On the other hand, $(PhSe)_2$ is thought to play a antioxidant role against cadmium toxicity, since this compound was effective in ameliorate both δ-ALA-D activity and TBARS levels at both doses of cadmium studied.

Another interesting observation in the present study is a marked inhibitory effect of cadmium treatment on testes SOD activity. However, treatment with both compounds DMSA and $(PhSe)_2$ and the combined therapy were ineffective in restoring SOD activity. Similarly, cadmium-treated mice had reduction on ascorbic acid content. Though, administration of $(PhSe)_2$ and DMSA, either individually or in combination, was unable to restore ascorbic acid to the normal values. These results indicate that neither monotherapy nor combined therapy was able to recover antioxidant defenses (an enzymatic (SOD) and a non-enzymatic (ascorbic acid)) modified by cadmium treatment.

In summary, the use of combined therapy (DMSA plus $(PhSe)_2$) did not proved to be better than the monotherapy, in improving toxicological parameters evaluated in this model of testicular damage induced by cadmium. However, the use of $(PhSe)_2$, an antioxidant, seems to be useful in ameliorating lipid peroxidation, a process important for cadmium toxicity.

Acknowledgements

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4.2.2 – **Artigo 4**

EFFICACY OF 2,3-DIMERCAPTO-1-PROPANESULFONIC ACID (DMPS) AND DIPHENYL DISELENIDE ON CADMIUM INDUCED TESTICULAR DAMAGE IN MICE

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Efficacy of 2,3-dimercapto-1-propanesulfonic acid (DMPS) and diphenyl diselenide on cadmium induced testicular damage in mice

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Abbreviated title: DMPS plus $(PhSe)_2$ on cadmium-induced testes damage in mice

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Abstract

The deleterious effect of acute cadmium-intoxication in mice testes was evaluated. Animals received a single dose of $CdCl_2$ (2.5 or 5 mg/kg, intraperitoneally) and a number of toxicological parameters in mice testes were examined such as δ-aminolevulinic acid dehydratase (δ-ALA-D) activity, lipid peroxidation, hemoglobin and ascorbic acid contents. Furthermore, the parameters that indicate tissue damage such as plasmatic alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were also determined. Thus, a possible protective effect of 2,3-dimercapto-1-propane-sulfonic acid (DMPS) and diphenyl diselenide $(PhSe)_2$ are studied. The results demonstrated inhibition of δ-ALA-D activity, reduction in ascorbic acid, increase of lipid peroxidation induced by cadmium, indicating testes damage. As well, we observed an increase on plasmatic LDH, AST and ALT activities. DMPS (400 μmol/Kg) and $(PhSe)_2$ (100 μmol/Kg) protected inhibitory effect of 2.5 mg/kg $CdCl_2$ on δ-ALA-D and restored the increase of TBARS levels. $(PhSe)_2$ therapy was effective in ameliorate ascorbic acid content when the cadmium dose was 2.5 mg/kg. Treatment with DMPS and $(PhSe)_2$, individually or after combined therapy, were inefficient in restoring plasmatic LDH and ALT activity at control level. The use of combined therapy (DMPS plus $(PhSe)_2$) proved be better than the monotherapy in decreasing cadmium levels in testes and in ameliorating plasmatic AST activity from animals that received the highest dose of cadmium.

Key- Words: Antioxidant; Cadmium; DMPS; Selenium; Organoselenium; Testes

1. Introduction

Cadmium (Cd) is an environmental contaminant with food and tobacco smoking being the main sources of exposure in the non-occupationally exposed population (WHO, 1992). Acute Cd poisoning produces primarily hepatic and testicular injury, whereas chronic exposure results in renal damage and osteotoxicity (Rikans et al., 2000). Testes can be particularly affected; a single carcinogenic dose of cadmium can cause significant testes pathology (hemorrhage, atrophy and calcification) (Koizumi and Li, 1992). The problem of prevention and therapeutic intervention in cadmium intoxication may be approached in two ways: (i) chelation of cadmium that has been localized intracellularly bound to metallothionein mainly in liver and kidney after the exposure (Goyer et al., 1995; Andersen, 1999); and (ii) free radical scavenging by antioxidants and enzymatic defence system (Farris, 1991; Hudecova and Ginter, 1992). In this way, several authors have shown that antioxidant should be one of the important components of an effective treatment of cadmium poisoning (Casalino et al., 2002; El-Demerdash et al., 2004). Furthermore, it is believed that the effects of combined therapy with antioxidants and chelators can yield better therapeutic outcomes than isolated chelation therapy (Pande and Flora, 2002, Tandon et al., 2003).

The sodium salt of 2,3-dimercapto-1-propane-sulfonic acid (DMPS, Dimaval®) is less toxic and more effective chelating agent than the chemically analogous dimercaprol (British Anti-Lewisite, BAL) (Aposhian et al., 1995; Soares et al., 2003). DMPS is registered in Germany for the treatment of mercury intoxication and has been used to treat mercury (Campbell et al., 1986) or lead poisoning in children and adults (Kemper et al., 1990).

The concept that selenium-containing molecules may be better nucleophiles (and therefore antioxidants) than classical antioxidants, has led to the design of synthetic organoselenium compounds (Arteel and Sies, 2001). In fact, several reports have been published on glutathione peroxidase (GSH-px)-mimetic seleno-compounds, which, like the native enzyme, rely on the redox cycling of selenium. Moreover, diphenyl diselenide $(PhSe)_2$ has been shown to be even more active as a glutathione peroxidase mimic (Meotti et al., 2004) and less toxic to rodents than ebselen (Nogueira et al. 2003a; Meotti et al. 2003).

The objective of the present study was to examine the beneficial effects of DMPS when given alone or in combination with $(PhSe)_2$ on Cd-induced testicular damage. Thereby, we evaluated the effect of Cd on δ-ALA-D activity, lipid peroxidation, ascorbic acid and hemoglobin content in mice testes. The parameters that indicate tissue damage like plasmatic AST, ALT and LDH were also determined.

2- Materials and methods

2.1- Chemicals

DMPS (2,3-dimercapto-1-propane-sulfonic acid), $CdCl_2$, δ-aminolevulinic acid (δ-ALA) and *p*-dimethylaminobenzaldehyde were purchased from Sigma (St. Louis, MO, USA). Diphenyl diselenide (Figure 1) 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. DMPS and $(PhSe)_2$ were dissolved in DMSO (dimethylsulfoxide).

2.2- Animals

Male adult Swiss albino mice (25-35g) 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, School of Medicine Veterinary and Animal Science of the University of Sao Paulo, Brazil.

2.3- Exposure:

A group of six to eight mice was usually tested in each experiment. The mice were injected intraperitoneally with a single dose of $CdCl_2$ (2.5 or 5 mg/kg) (dissolved in saline at 0.25 and 0.5 mg/mL) and 30 min later they were injected intraperitoneally with 400 μmol/kg DMPS (Andersen, 1989) or subcutaneously with diphenyl diselenide (100 μmol/kg), an effective and non-toxic dose (Nogueira et al., 2003b). The cadmium intoxication protocol has been chosen based on published papers (Min et al., 2002, Santos et al., 2004) in an attempt to cause a severe testicular injury. The testicular damage was evidenced by increase of hemoglobin content and TBARS levels as described by Koizumi and Li (1992).

Animals were slight anesthetized for blood collect 24 h after $CdCl_2$ treatment and then, testes were removed. The protocol of mice treatment is given below:

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

Group 2 - $CdCl_2$ (2.5 mg/kg, i.p.) + DMSO (s.c.) + DMSO (i.p.)

Group 3 - $CdCl_2$ (5 mg/kg, i.p.) + DMSO (s.c.) + DMSO (i.p.)

Group 4 – saline (i.p.) + DMSO (s.c.) + DMPS (400 μmol/kg, i.p.)

Group 5 – saline (i.p.) + $(PhSe)_2$ (100 μmol/kg, s.c.) + DMSO (i.p.)

Group 6 - saline (i.p.) + DMPS (400 μmol/kg, i.p.) + $(PhSe)_2$ (100 μmol/kg, s.c.)

Group 7 - $CdCl_2$ (2.5 mg/kg, i.p.) + DMSO (s.c.) + DMPS (400 μmol/kg, i.p.)

Group 8 - $CdCl_2$ (2.5 mg/kg, i.p.) + $(PhSe)_2$ (100 μmol/kg, s.c.) + DMSO (i.p.)

Group 9 - $CdCl_2$ (2.5 mg/kg, i.p.) + DMPS (400 μmol/kg, i.p.) + $(PhSe)_2$ (100 μmol/kg, s.c.)

Group 10 - $CdCl_2$ (5 mg/kg, i.p.) + DMSO (s.c.) + DMPS (400 μmol/kg, i.p.)

Group 11 - $CdCl_2$ (5 mg/kg, i.p.) + $(PhSe)_2$ (100 μmol/kg, s.c.) + DMSO (i.p.)

Group 12 - $CdCl_2$ (5 mg/kg, i.p.) + DMPS (400 μmol/kg, i.p.) + $(PhSe)_2$ (100 μmol/kg, s.c.)

2.4- Lipid peroxidation

Testes were rapidly homogenized in 50 mM Tris-Cl, pH 7.5 (1/10, w/v) and centrifuged at 2,400 *x* g for 15 min. An aliquot (200 μL) of homogenized was incubated at 95^0C for 2 hours. TBARS was determined as described by Ohkawa et al. (1979).

2.5- Ascorbic acid determination

Ascorbic acid determination was performed as described by Jacques-Silva et al. (2001). Proteins (testes) were precipitated in 10 volumes of a cold 4 % trichloroacetic acid solution. An aliquot of the sample in a final volume of 1 ml of the solution was incubated for 3 hr at 38°C then 1 mL H_2SO_4 65 % (v/v) was added to the medium. The reaction product was determined using color reagent contained 4.5 mg/ml dinitrophenyl hydrazine and $CuSO_4$ (0.075 mg/mL). The content of ascorbic acid is related to tissue amount (μmol ascorbic acid/g wet tissue).

2.6 -δ-Aminolevulinic dehydratase(δ-ALA-D) activity

Testicular δ-ALA-D activity was assayed according to the method of Sassa (1982) by measuring the rate of product porphobilinogen (PBG) formation except that 45 mM sodium phosphate buffer and 2.2 mM δ-ALA were used. Samples were homogenized in 0.9% NaCl in the proportion (w/v) 1/5 and centrifuged at 2,400 *x* g for 15 min. An aliquot of 50 μL of homogenized tissue was incubated for 2h at 37 ^{0}C. Reaction was linear in relation to protein and time of incubation. The reaction product was determined using modified Erlich's reagent at 555 nm

2.7- Hemoglobin content and protein determination

Hemoglobin concentration in testes supernatants was assayed using a commercial Kit (LABTEST Diagnostica S.A., Minas Gerais, Brazil) according to the method of Henry et al., (1974). Protein was measured by the Coomassie blue method according to Bradford (1976) using bovine serum albumin as standard.

2.8- Plasmatic transaminases (AST and ALT) and Lactate dehydrogenase (LDH) activities

Plasmatic AST and ALT enzymes were used as biochemical markers for the early acute hepatic damage and determined by the colorimetric method of Reitman and Frankel (1957) (LABTEST, Diagnostica S.A., Minas Gerais, Brazil).

LDH activity was monitored spectrophotometrically by the rate of increase in absorbance at 340 nm at 30°C resulting from formation of NADH (Pereira et al., 1991). 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 pre incubated 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.

2.9- Metal determination

Concentration of cadmium in testes was determined by graphite furnace atomic absorption spectrometry using a Varian SpectrAA 200 spectrometer (Melbourne, Australia). The samples were digested with a mixture of HNO_3 conc. and $HClO_4$ conc. 3+1 (5mL) and diluted to 20 mL before measuring their cadmium content (Welz et al., 1999).

2.10- Statistical analysis

Data are expressed as means±S.E.M. Statistical analysis was performed using a three-way analysis of variance (ANOVA), followed by the 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. For cadmium determination, a non-parametric Kruskal-Wallis test, followed by Mann-Whitney U test was applied because control group had no variance (all values were bellow the detection limit).

3- Results

3.1- Lipid peroxidation

Three-way ANOVA of TBARS levels yield a significant DMPS x $(PhSe)_2$ x Cd^{2+} interaction. Post-hoc comparisons demonstrated that 2.5 and 5 mg/kg Cd^{2+} increased ($p<0.0001$) lipid peroxidation in testes (4.38- and 5-fold higher, respectively, than the corresponding control values). $(PhSe)_2$ treatment was effective in restoring increased TBARS caused by 2.5 mg/kg cadmium to control level. However, $(PhSe)_2$ was ineffective in restoring TBARS status towards to control level when mice received 5 mg/kg cadmium. DMPS administration and concomitant therapy with DMPS plus $(PhSe)_2$ were effective in reducing cadmium-induced increase on TBARS in testes, when the cadmium dose was 2.5 mg/kg (Figure 2).

3.2- Ascorbic acid determination

Three-way ANOVA of ascorbic acid content yielded a significant main effect of Cd^{2+} ($p<0.0001$). Post-hoc comparison demonstrated that acute cadmium exposure, at two tested doses (2.5 and 5 mg/kg), significantly reduced ascorbic acid levels (59 and 70 %, respectively) in testes ($p<0.0001$). $(PhSe)_2$ therapy was effective in ameliorating ascorbic acid content when the cadmium dose was 2.5 mg/kg, but levels of ascorbic acid reached only ~50% of control levels. Ascorbic acid levels remained unchanged after treatment with DMPS, individually or after combined treatment with $(PhSe)_2$ (Figure 3).

3.3- δ-ALA-D activity

Three-way ANOVA of testicular δ-ALA-D activity yielded a significant $(PhSe)_2$ x Cd^{2+} interaction ($p<0.05$). Post-hoc comparisons demonstrated that acute cadmium exposure, at two tested doses (2.5 and 5 mg/kg), significantly inhibited δ-ALA-D activity (49 and 56 %, respectively) in testes ($p<0.0001$). $(PhSe)_2$ and DMPS individually or combined, $(PhSe)_2$ plus DMPS, eliminated inhibition on δ-ALA-D activity caused by cadmium at both studied doses (Figure 4).

3.4- Hemoglobin concentration

Three-way ANOVA of testicular hemoglobin yielded a significant main effect of Cd^{2+} ($p<0.0001$). Post-hoc comparisons indicated that cadmium treatment was associated with a significant accumulation of hemoglobin in testes ($p<0.0001$), which was 2.4-times (2.5 mg/kg Cd^{2+} group) and 3.2-times (5 mg/kg Cd^{2+}group) higher than in the control group (Figure 5). $(PhSe)_2$, DMPS or their combination were unable to restore testes hemoglobin content at normal level.

3.5- Lactate dehydrogenase (LDH) activity

Three-way ANOVA of LDH activity yielded a significant DMPS x Cd^{2+} interaction ($p<0.05$). Mice exposed to 2.5 mg/kg cadmium did not present change on plasma LDH activity. Post-hoc comparisons demonstrated that 5 mg/kg Cd^{2+} intoxication caused a increase of about 1.87-times on LDH activity ($p<0.0001$). $(PhSe)_2$, DMPS or their combination significantly decreased the enzyme activity, however, these compounds were ineffective in restoring LDH activity at control level (Table 1).

3.6- Transaminase activity

Three-way ANOVA of ALT activity yielded a significant main effect of Cd^{2+} ($p<0.0001$). Post-hoc comparisons indicated that cadmium treatment was associated with a significant increase on plasmatic ALT ($p<0.0001$), which was 2.58-times (2.5 mg/kg Cd^{2+} group) and 3.7-times (5 mg/kg Cd^{2+}group) higher than in the control group (Table 2). Treatment with DMPS and $(PhSe)_2$, individually or after combined treatment, were inefficient in restoring ALT levels towards the control level. Three-way ANOVA of AST activity yielded a significant $(PhSe)_2$ x Cd^{2+} interaction ($p<0.05$). Similarly, 2.5 and 5.0 mg/kg cadmium exposure showed a significant increase on AST activity ($p<0.0001$), around of 1.58- and 2-fold, respectively. The concomitant therapy, DMPS plus $(PhSe)_2$, was effective in ameliorating enzyme activity when the dose of cadmium was 5.0 mg/kg, but not at control level (Table 2).

3.7- Cadmium determination

Testes cadmium accumulation following the doses of 2.5 and 5.0 mg/kg cadmium was higher (264- and 736-fold, respectively) than in control mice (Table 3). Furthermore, animals that received dose of 5 mg/kg accumulated significantly more cadmium in mice testes than those that received dose of 2.5 mg/kg. Treatment with DMPS and $(PhSe)_2$,

individually or after combined treatment, were inefficient in restoring cadmium levels towards the control level when the cadmium dose was 2.5 mg/kg.

In mice exposed to 5.0 mg Cd/kg, cadmium levels on testes were decreased after treatment with DMPS and $(PhSe)_2$ and the concomitant treatment with DMPS and $(PhSe)_2$ was more efficient in decreasing cadmium levels than monotherapy ($p<0.05$) (Table 5).

4- Discussion

Cadmium is one of the most abundant non-essential elements due to its immense usage in various industrial applications (Page et al., 1986). The health risk to humans from cadmium intoxication and the toxicity from acute and chronic exposure have been well described (Goyer, 1996). In addition, cadmium promotes an early oxidative stress and afterward contributes to the development of serious pathological conditions because of its long retention in some tissues (Bagehi et al., 2000). Therefore, protection against the acute actions of cadmium can be achieved through the antioxidant systems (Rana and Verma, 1996).

Acute cadmium poisoning produces primarily hepatic and testicular injury (Rikans et al., 2000, Santos et al, 2004). Accordingly, we demonstrated, in this study, that acute administration of cadmium causes a marked increase in TBARS production and hemoglobin, as well a decrease on δ-ALA-D activity and ascorbic acid content in mice testes. Furthermore, a significant increase in plasmatic LDH, ALT and AST activity was observed.

In fact, cadmium-treated groups demonstrated high hemoglobin content, which is in agreement with the visual observation of testes hemorrhagic infiltration. The use of monotherapy (DMPS and $(PhSe)_2$) or the combined therapy was inefficient in ameliorating

hemorrhage caused by cadmium. Plasmatic LDH activity was also found enhanced indicating tissue damage, probably a testicular damage. Therapy with DMPS and $(PhSe)_2$, or combined therapy unchanged LDH activity. Curiously, DMPS and $(PhSe)_2$, *per se*, reduced significantly plasmatic LDH activity.

In addition, cadmium exposed-mice presented an increase in plasmatic AST and ALT activities that could be mainly due to the leakage of these enzymes from the liver cytosol into the blood stream (Navarro et al., 1993) suggesting hepatotoxic effect of cadmium. According to some authors (Rana et al., 1996; El-Demerdash et al., 2004), the increase on plasmatic AST and ALT activities could also indicate a decrease on liver enzyme activity. Concerning therapy, DMPS and $(PhSe)_2$ was not efficient in changing AST and ALT activities, however, concomitant therapy (DMPS plus $(PhSe)_2$) was effective in restoring AST activity induced by 5.0 mg/kg cadmium.

Lipid peroxidation has long been considered the primary mechanism for cadmium toxicity (Manca et al., 1991), despite its inability to directly generate free radicals under physiological conditions (Eneman et al., 2000). Thus, it is believed that antioxidant should be one important component of an effective treatment of cadmium poisoning (Casalino et al., 2002; El-Demerdash et al., 2004) suggesting that the effects of combined therapy with antioxidants and chelators can yield better therapeutic outcomes than isolated chelation therapy (Tandon et al., 2003).

In this study, $(PhSe)_2$ therapy was effective in restoring TBARS enhance caused by 2.5 mg/kg cadmium, as well as, concomitant treatment (DMPS plus $(PhSe)_2$). The therapy with $(PhSe)_2$ alone seems to have better effect on recovery of increased TBARS levels at both cadmium doses than the combined therapy ($(PhSe)_2$ plus DMPS); however, there is no

significant difference between these groups. Recently, we demonstrated that DMSA was effective in ameliorated testes TBARS levels in mice exposed to 2.5 mg/kg cadmium (Santos et al., 2004). In contrast, even causing reduction on TBARS levels, DMPS therapy was not effective in improving this parameter towards the control level. Moreover, $(PhSe)_2$ therapy was effective in ameliorated ascorbic acid reduction caused by 2.5 mg/kg cadmium, whereas DMPS was not efficient on this parameter.

Recently, we demonstrated changes in testicular δ-ALA-D activity after cadmium exposure (Santos et al., 2004, Folmer et al., 2004) and for this ending point of toxicity, $(PhSe)_2$ and DMPS were effective in restoring δ-ALA-D activity to control levels. The exact mechanism to explain the enzyme inhibition is yet unclear, tentatively cadmium-induced inhibitory effect could be related to lipid peroxidation since δ-ALA-D activity is extremely sensitive to situations associated with oxidative stress (Pande and Flora, 2002, Soares et al, 2003, Nogueira et al., 2004). Other hypothesis for enzyme inhibition is the fact that mammalian δ-ALA-D is a metalloenzyme that requires Zn^{2+} for maximal catalytic activity (Nogueira et al., 2003a, 2003c), thus Cd^{2+} could cause a Zn^{2+} displacement leading to enzyme inhibition.

The cadmium dose of 5 mg/kg accumulated significantly more cadmium in mice testes than 2.5 mg/kg. Therapy with DMPS and $(PhSe)_2$, in mice exposed to 5.0 mg/kg, reduced cadmium levels, whereas the concomitant treatment with these compounds was more efficient in decreasing cadmium levels than monotherapy. This result is important from the toxicological viewpoint because diphenyl diselenide could be useful to help on cadmium detoxification. Regarding this matter, we presented a hypothesis that could help to explain diphenyl diselenide and DMPS ability to reduce cadmium levels. Thus, cadmium

detoxification is believed to follow a catalytic mechanism, since there is not an equimolar relation between Cd/DMPS and Cd/diphenyl diselenide, even considering that each molecule of diphenyl diselenide ($2PhSe^-$) or DMPS ($2RS^-$) could react with 2 atoms of Cd. Unexpectedly, combined treatment (DMPS plus $(PhSe)_2$ was similar to montherapy in decreasing cadmium levels in animals exposed to 2.5 mg/kg cadmium.

In conclusion, the results suggest that the use of combined therapy (DMPS plus $(PhSe)_2$) proved to be better than the monotherapy in decreasing cadmium levels in testes and in ameliorating plasmatic AST activity from animals that received greatest dose of cadmium, whereas combined therapy failed in improving other toxicological parameters evaluated in this model of testicular damage induced by cadmium. In addition, the use of $(PhSe)_2$ as an antioxidant seems to be useful in reducing cadmium levels and in ameliorating lipid peroxidation, a process important for cadmium toxicity.

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Tables

Table 1-Plasmatic lactate dehydrogenase (LDH) activity of mice exposed to 2.5 and 5 mg/kg cadmium

Groups	Control	Cd 2.5	Cd 5.0
Control	232.60 ± 79	302.50 ± 95	435.50 ± 85*
DMPS	64.33 ± 28*	293.33 ± 85	450.00 ± 14*#
$(PhSe)_2$	53.33 ± 23*	175.00 ± 7#	340.00 ± 72
$DMPSx(PhSe)_2$	61.25 ± 16*	363.33 ± 112*	400.00 ± 46*

Data are expressed as IU/L. Data are mean±S.D. from six animals in each group. (*) Denoted $p<0.0001$ as compared to control group (three-way ANOVA/Duncan). (#) Denoted $p<0.05$ as compared to Cd 2.5 group.

Table 2- Plasmatic alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities of mice exposed to 2.5 and 5 mg/kg cadmium

Enzyme activity	ALT			AST		
Groups	Control	Cd 2.5	Cd 5.0	Control	Cd 2.5	Cd 5.0
Control	100	258±147*	371±106*#	100	158 ± 32*	203 ± 47*
DMPS	100 ± 22	165 ± 14	335 ± 87*#	82 ± 13	145 ± 25	169 ± 23*
$(PhSe)_2$	98 ± 22	166 ± 30	406 ± 51*#	94 ± 24	166 ± 30*	168 ± 16*
$DMPSx(PhSe)_2$	103 ± 27	180 ± 58	317±100*#	79 ± 5	180 ± 58*	133 ± 8§

Data are expressed as % of Control. ALT and AST activities of control (100 %) was 24.28 ± 2.7 and 46.25 ± 2.5 IU/L, respectively. Data are mean±S.D. from six animals in each group. (*) Denoted $p<0.0001$ as compared to control group (three-way ANOVA/Duncan). (#) Denoted $p<0.05$ as compared to Cd 2.5 group. (§)Denoted $p<0.05$ as compared to Cd 5.0 group.

Table 3- Cadmium concentration in testes of mice exposed to 2.5 and 5 mg/kg cadmium

Groups	Control	Cd 2.5	Cd 5.0
Control	< 0.2	52.82± 4*	147.26 ± 18*#
DMPS	< 0.2	34.72 ± 0.8*	118.44 ± 16*#§
$(PhSe)_2$	< 0.2	41.33 ± 4*	118.39 ± 35*#§
$DMPSx(PhSe)_2$	< 0.2	38.38 ± 0.7*	85.55 ± 32*#§$

Data are expressed as μg Cd/g. Data are mean±S.D. from six animals in each group.
(*) Denoted $p<0.0001$ as compared to control group by Kruskal-Wallis, followed by Mann-Whitney U test.
(#) Denoted $p<0.05$ as compared to Cd 2.5 group by Kruskal-Wallis, followed by Mann-Whitney U test.
(§) Denoted $p<0.05$ as compared to Cd 5.0 group by Kruskal-Wallis, followed by Mann-Whitney U test.
($) Denoted $p<0.05$ as compared to all other experimental groups by Kruskal-Wallis, followed by Mann-Whitney U test.
< 0.2 was the limit of detection for cadmium determination.

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Legends

Figure 1- Diphenyl diselenide

Figure 2- Effect of $(PhSe)_2$, DMPS or their combination on cadmium-induced alterations in TBARS levels in testes of cadmium-exposed mice. Data are reported as mean±S.E.M. of seven animals per group and expressed as nmol MDA (malondialdehyde)/g wet tissue. (*) Denoted $p<0.05$ as compared to control group (three-way ANOVA/Duncan). (#) Denoted $p<0.05$ as compared to Cd 2.5 group.

Figure 3- Effect of $(PhSe)_2$, DMPS or their combination on cadmium-induced alterations in ascorbic acid levels in testes of cadmium-exposed mice. Data are reported as mean±S.E.M. of seven animals per group. (*) Denoted $p<0.05$ as compared to control group (three-way ANOVA/Duncan). (#) Denoted $p<0.05$ as compared to Cd 2.5 group.

Figure 4- Effect of $(PhSe)_2$, DMPS or their combination on cadmium-induced alterations in δ-ALA-D activity in testes of cadmium-exposed mice. Tissues were pre-incubated at 37ºC for ten minutes. Enzymatic reaction was initiated by adding the substrate (ALA) to a final concentration of 2.2 mM in a medium containing 45 mM phosphate buffer, pH 6.8. Data

are reported as mean±S.E.M. of seven animals per group and expressed as nmol porphobilinogen (PBG)/ mg protein/ hour. (*) Denoted $p<0.05$ as compared to control group (three-way ANOVA/Duncan).

Figure 5- Effect of $(PhSe)_2$, DMPS or their combination on cadmium-induced alterations in hemoglobin concentration in testes of cadmium-exposed mice. Data are reported as mean±S.E.M. of seven animals per group. (*) Denoted $p<0.05$ as compared to control group (three-way ANOVA/Duncan).

Figures

Figure 1

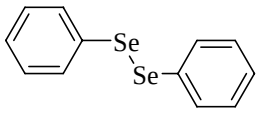


Figure 2

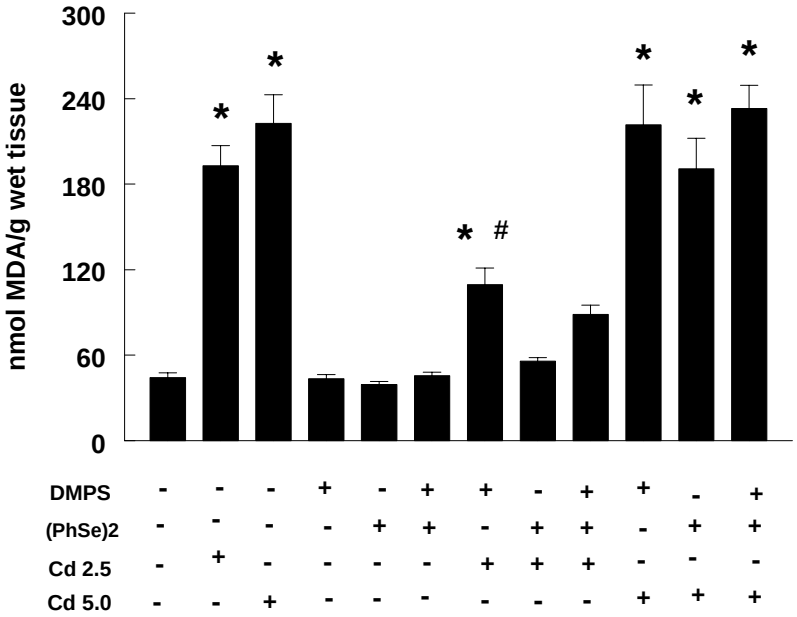


Figure 3

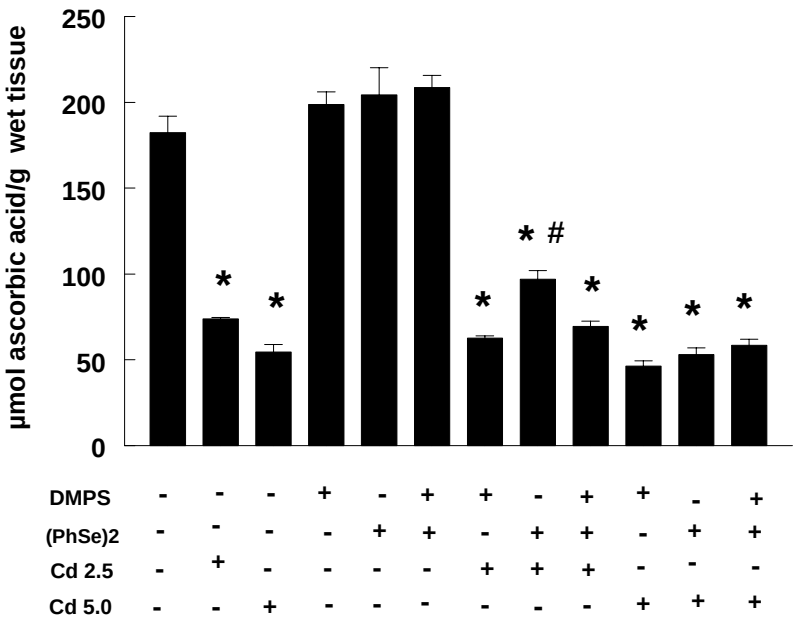


Figure 4

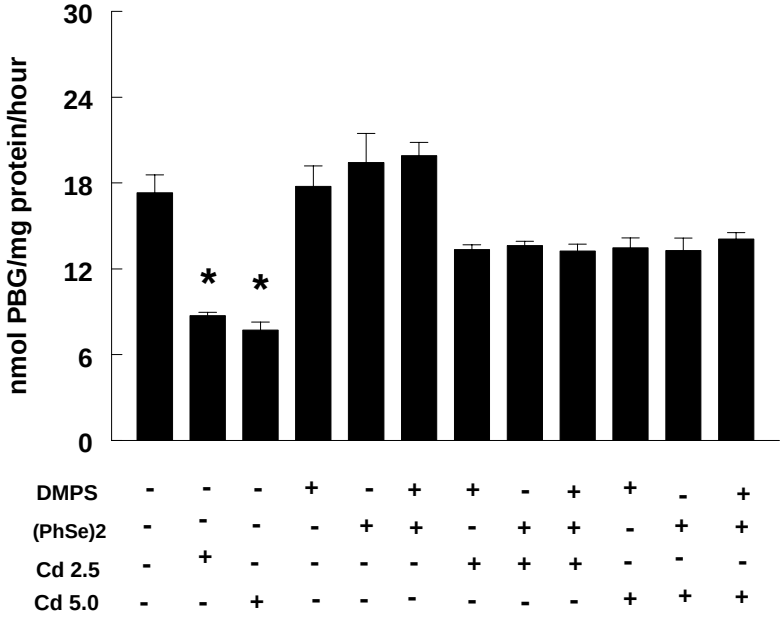
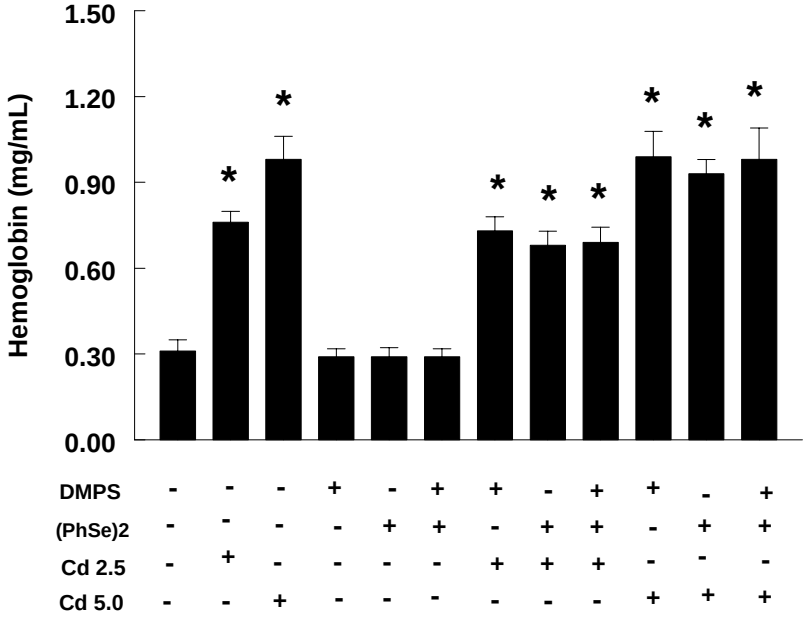


Figure 5



4.2.3 – **Artigo 5**

DIPHENYL DISELENIDE REVERSES CADMIUM-INDUCED OXIDATIVE DAMAGE ON MICE TISSUES

Chemico-Biological Interactions, *in press* (2005)

Diphenyl Diselenide Reverses Cadmium-Induced Oxidative Damage on Mice Tissues

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Abbreviated title: $(PhSe)_2$ on cadmium-induced oxidative damage in mice

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Abstract

The concept that selenium-containing molecules may be better antioxidants than classical antioxidants, has led to the design of synthetic organoselenium compounds. In the present investigation subchronic deleterious effects of cadmium-intoxication in mice and a possible protective effect of diphenyl diselenide $(PhSe)_2$ (5 μmol/Kg) were studied. Male adult Swiss albino mice (25-35g) received $CdCl_2$ (10 μmol/Kg, subcutaneously), 5 times/week, for 4 weeks. A number of toxicological parameters in blood, liver, kidney, spleen and brain of mice were examined including δ-aminolevulinic acid dehydratase (δ-ALA-D) activity, lipid peroxidation and ascorbic acid content, the parameters that indicate tissue damage such as plasmatic alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea, creatinine and lactate dehydrogenase (LDH) were also determined. The results demonstrated that cadmium caused inhibition of δ-ALA-D activity in liver (24 %), kidney (33 %) and spleen (73 %) and $(PhSe)_2$ therapy was effective in restoring enzyme activity in all tissues. A reduction in ascorbic acid content was observed in kidney (11 %) and spleen (10.7 %) of cadmium-treated mice and $(PhSe)_2$ was only effective in improving this reduction in kidney. An increase of lipid peroxidation induced by cadmium was noted in liver (29 %) and brain (28 %) tissues and $(PhSe)_2$ therapy was effective in restoring TBARS levels in both tissues. We also observed an increase on plasmatic LDH (1.99-times), AST (1.93-times) and ALT (4.24-times) activities and $(PhSe)_2$ therapy was effective in restoring AST activity at control level. This compound had not a significant effect on plasmatic parameters evaluated, indicating that $(PhSe)_2$ not present toxic effects in studied dose. The results suggest that the administration of an antioxidant $(PhSe)_2$ during cadmium intoxication may provide beneficial effects by reducing oxidative stress in tissues.

Key- Words: Antioxidant; Cadmium; Selenium; Organoselenium; Mice

1. Introduction

The exposure of human populations to a variety of heavy metals has been a public health concern [1]. Cadmium (Cd) is one of the most abundant non-essential elements due to its immense usage in various industrial applications [2]. Therefore, human intoxication has been investigated following occupational as well as environmental exposure [3]. The molecular mechanism responsible for the toxic effects of cadmium is far from being completely understood. However, lipid peroxidation has long been considered the primary mechanism for cadmium toxicity [4, 5, 6, 7]. Thus, it is believed that antioxidant should be one of the important components of an effective treatment of cadmium poisoning. In line with this, several studies have been performed with different natural substances possessing antioxidant properties to investigate their possible protective effects in cadmium-induced tissues damage. Among those melatonin, α-lipoic acid, quercetin, hydroxytyrosol and coenzyme Q10 have been addressed to have protective functions [8, 9, 10, 11, 12].

The concept that selenium-containing molecules may be better nucleophiles (and therefore antioxidants) than classical antioxidants, has led to the design of synthetic organoselenium compounds [13]. Several reports have been published on glutathione peroxidase (GSH-px)-mimetic seleno-compounds, which, like the native enzyme, rely on the redox cycling of selenium. In fact, recent study has shown that the diaryl diselenides were potent antioxidants in mice [14]. A variety of seleno organic compounds are now considered as potential pharmacological agents [15, 16, 17, 18].

Recently, we demonstrated that diphenyl diselenide was as effective in restoring acute cadmium-induced oxidative damage in mice testes as the chelating compounds succimer [6] and DMPS [7]. Thus, in the present study we investigated the beneficial effects of diphenyl diselenide on subchronic cadmium-poisoning. Thereby, we evaluated the effect of cadmium on toxicological parameters in mice tissues.

2- Materials and methods

2.1- Chemicals

$CdCl_2$, δ-aminolevulinic acid (δ-ALA) and *p*-dimethylaminobenzaldehyde were purchased from Sigma (St. Louis, MO, USA). Diphenyl diselenide (Figure 1) was synthesized according to Paulmier [19]. 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).

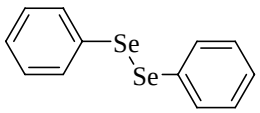

Figure 1- Diphenyl diselenide

2.2- Animals

Male adult Swiss albino mice (25-35g) from our own breeding colony were used. The animals were kept on separate animal rooms, on a 12 hour 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, School of Medicine Veterinary and Animal Science of the University of Sao Paulo, Brazil.

2.3- Experimental Design:

Groups of six to eight mice were usually tested in each experiment. Mice received cadmium chloride ($CdCl_2$), subcutaneously, at 10 μmol/kg dose (dissolved in saline at 0.25 mg/ml), 5 times/week, for 4 weeks [20]. Thirty min later they were injected subcutaneously with diphenyl diselenide (5 μmol/kg), an effective and non-toxic dose [17]. Animals were slight anesthetized for blood collect 24 h after the last $CdCl_2$ treatment and liver, kidney, brain and spleen were removed.

Table 1 - Protocol of mice treatment

Groups	Treatments
Group 1 - (n=6)	Saline (s.c.) + DMSO (s.c.)
Group 2 - (n=8)	$CdCl_2$ (10 μmol/kg, s.c.) + DMSO (s.c.)
Group 3 - (n=6)	Saline (s.c.) + $(PhSe)_2$ (5 μmol/kg, s.c.)
Group 4 - (n=8)	$CdCl_2$ (10 μmol/kg, s.c.) + $(PhSe)_2$ (5 μmol/kg, s.c.)

Animals received the treatments during 4 weeks, 5 times/week. "n" represents the number of mice tested in each group.

2.4- Lipid peroxidation

Tissues were rapidly homogenized in 50 mM Tris-Cl, pH 7.5 (1/10, w/v) and centrifuged at 2,400 *x* g for 15 min. An aliquot (200 μL) of homogenate was incubated at 95^0C for 2 hours. TBARS was determined as described by Ohkawa et al. [21].

2.5- Ascorbic acid determination

Ascorbic acid determination was performed as described by Jacques-Silva et al. [22]. Protein (tissues) was precipitated in 10 volumes of a cold 4 % trichloroacetic acid solution.

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

2.6 -δ-Aminolevulinic dehydratase(δ-ALA-D) activity

δ-ALA-D activity of tissues was assayed according to the method of Sassa [23] by measuring the rate of product (porphobilinogen) formation except that 45 mM sodium phosphate buffer and 2.2 mM δ-ALA were used. Samples were homogenized in 0.9% NaCl in the proportion (w/v) 1/5 and centrifuged at 2,400 *x* g for 15 min. An aliquot of 200 μL of homogenized tissue was incubated for 0.5h (liver), 1h (kidney and spleen) and 3h (brain) at 37 ^{0}C. The reaction product was determined using modified Erlich's reagent at 555 nm.

2.7- Plasma transaminases (AST and ALT), lactate dehydrogenase (LDH) activities and urea, creatinine levels

Plasmatic enzymes AST and ALT were used as the biochemical markers for the early acute hepatic damage [24], using a commercial Kit (LABTEST, Diagnostica S.A., Minas Gerais, Brazil). Renal function was analysed using a commercial Kit (LABTEST, Diagnostica S.A., Minas Gerais, Brazil) by determining plasmatic urea [25] and creatinine [26]. LDH activity was monitored spectrophotometrically by the rate of increase in absorbance at 340 nm at 30°C resulting from formation of NADH [27]. 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.

2.9- Statistical analysis

Data are expressed as means±S.D. Statistical analysis was performed using a one-way analysis of variance (ANOVA), followed by the Duncan's multiple range test when appropriate. Values of $p<0.05$ were considered statistically significant.

3- Results

3.1- Lipid peroxidation

Cadmium (10 μmol/kg) increased lipid peroxidation in liver ($p<0.005$) and brain ($p<0.05$) (1.29- and 1.28-fold higher, respectively, than control group) and $(PhSe)_2$ treatment was effective in restoring TBARS enhance caused by cadmium in these tissues. Surprisingly, cadmium caused a reduction on TBARS levels in spleen ($p<0.05$) and kidney ($p<0.0001$), whereas $(PhSe)_2$ treatment did not change the decrease caused by cadmium intoxication (Table 2).

3.2- Ascorbic acid determination

Subchronic exposure with cadmium significantly reduced ascorbic acid levels in kidney ($p<0.05$) and spleen ($p<0.05$) (11 and 10.7 %, respectively). $(PhSe)_2$ therapy was effective in restoring kidney ascorbic acid content at control level in cadmium exposed mice. Decreased ascorbic acid levels in spleen remained unchanged after $(PhSe)_2$ treatment. A decrease in ascorbic acid levels was observed in mice kidney that received only $(PhSe)_2$ treatment ($p<0.05$). The ascorbic acid levels were not change in liver and brain after cadmium exposure (Table 3).

3.3- δ-ALA-D activity

Subchronic cadmium exposure significantly inhibited δ-ALA-D activity in liver ($p<0.0001$), kidney ($p<0.0001$) and spleen ($p<0.001$) (24, 33 and 73 %, respectively). $(PhSe)_2$ restored inhibition on δ-ALA-D activity caused by cadmium in these tissues. Cadmium exposure did not change δ-ALA-D activity in brain (Table 4).

3.4- Plasmatic transaminases (AST and ALT), lactate dehydrogenase (LDH) activities and urea, creatinine levels

Cadmium treatment was associated with a significant increase on plasmatic ALT ($p<0.001$), which was 4.24-times higher than in control group (Table 5). Treatment with $(PhSe)_2$ was inefficient in restoring ALT level towards control group. Similarly, cadmium exposure showed a significant increase on AST activity ($p<0.005$), around of 1.93-fold. Therapy with $(PhSe)_2$ was effective in restoring enzyme activity at control group (Table 5).

Intoxication with cadmium caused an increase of about 1.99-times on LDH activity ($p<0.05$). $(PhSe)_2$ was ineffective in restoring LDH activity at control group (Table 5). Plasmatic urea and creatinine levels were not changed after subchronic cadmium poisoning.

4- Discussion

Cadmium is a toxic metal that is used in different industries. It promotes an early oxidative stress and afterward contributes to the development of serious pathological conditions because of its long retention in some tissues [28]. Therefore, protection against the acute actions of cadmium can be achieved through the antioxidant systems [29, 30].

Lipid peroxidation has long been considered the primary mechanism for cadmium toxicity [4, 5, 31], despite its inability to directly generate free radicals under physiological

conditions [32]. Thus, it is believed that antioxidant should be one of the important components of an effective treatment of cadmium poisoning [11, 33].

As far as we know, this is the first evidence that $(PhSe)_2$ has an antioxidant activity in mice tissues following subchronic cadmium intoxication. In fact, the present study clearly demonstrates the ability of not only cadmium to induce oxidative stress but also diphenyl diselenide therapy to restore enhance of TBARS levels caused by subchronic cadmium exposure in liver and brain of mice (Table 2). Recently, in a closely related investigation, we have demonstrated that $(PhSe)_2$ therapy was efficient in ameliorating oxidative stress on testes induced by a single cadmium administration in mice [6, 7].

Unexpectedly, TBARS levels were markedly reduced in kidney and spleen of cadmium intoxicated mice, in this study. The reasons for this decrease are still unclear to us but may be related to properties of kidney in accumulating cadmium, which competes with iron ions as initiator of lipoperoxidation. Furthermore, we also observed an decrease on ascorbic acid levels in kidney and spleen after cadmium poisoning, whereas $(PhSe)_2$ therapy was effective in restoring this antioxidant defense in kidney. Diphenyl diselenide was not effective at restoring ascorbic acid in the spleen as it was in the kidney.

In addition, we observed a decrease in hepatic, renal and spleenic δ-ALA-D activity and $(PhSe)_2$ therapy was effective in restoring enzyme activity in all studied tissues (Table 4). Since we have reported that δ-ALA-D activity is extremely sensitive to situations associated with oxidative stress [34, 35, 36, 37], the beneficial effect of $(PhSe)_2$ on this enzyme demonstrates, the antioxidant capacity of this compound. Actually, cadmium-induced inhibitory effect on hepatic δ-ALA-D activity can be tentatively related to lipid peroxidation on this tissue. We can also infer that δ-ALA-D inhibition participates, at least

in part, in the subchronic toxicity manifestations caused by cadmium. In contrast, cadmium administered in mice inhibited renal and hepatic δ-ALA-D activity but this effect is probably independent of thiobarbituric acid-reactive substance (TBARS) formation. Furthermore, mammalian δ-ALA-D is a metalloenzyme that requires zinc for maximal catalytic activity and data support the hypothesis of a direct competition between bivalent metals and zinc on δ-ALA-D from mammals [38, 39, 40]. Thus, cadmium (Cd^{2+}) could cause a zinc (Zn^{2+}) displacement leading to δ-ALA-D inhibition.

Cadmium exposed-mice presented an increase in plasmatic AST and ALT activities that could indicate a decrease in liver enzymes activity. In fact, Rana et al. [30] and El-Demerdash et al. [33] have reported that cadmium caused alterations in transaminases of rats. Therefore, the increase on the plasmatic activities of AST and ALT could be mainly due to the leakage of these enzymes from the liver cytosol into the blood stream [41], which could give an indication of the hepatotoxic effect of cadmium. Diphenyl diselenide therapy was efficient in restoring enhance of AST, but not ALT, activity induced by cadmium. Plasmatic LDH level, an indicator of tissue damage, was also increased on cadmium-exposed mice and remained unchanged after treatment with $(PhSe)_2$ treatment.

Besides, our data demonstrated that cadmium-exposure did not change urea and creatinine levels. This can be explained by the fact that cadmium is deposited primarily in the liver, where it induces and binds to metallothionein (MT) [42]. Over time, the hepatic CdMT is slowly released into the circulation and, subsequently, the protein is filtered from the blood by the renal glomeruli where it is degraded [43]. Thus, cadmium ions released from CdMT degradation bind to pre-existing renal MT and to newly synthesized MT, whereas excess of non-MT-bound cadmium is believed to cause nephrotoxicity [44],

presumably by generating free radicals. Since in this study there was a short time cadmium-exposure, perhaps it explains the hepatotoxicity and the absence of renal damage.

In conclusion, the results suggest that the use of $(PhSe)_2$ as an antioxidant seems to be useful in therapy of cadmium poisoning, since it has the capability to alleviate many of the harmful effects of cadmium.

Acknowledgements: The financial support by FAPERGS, CAPES and CNPq is gratefully acknowledged.

Table 2 - Effect of subchronic treatment with cadmium and $(PhSe)_2$ therapy on lipid peroxidation in mice liver, kidney, brain and spleen

TBARS (nmol MDA/mg protein)				
Group	Liver	Kidney	Brain	Spleen
Control	100.8 ± 9.6	20.9 ± 2.8	155.5 ± 85.0	37.2 ± 8.0
$(PhSe)_2$	100.5 ± 9.6	19.1 ± 3.1	161.4 ± 38.7	32.9 ± 6.3
Cd	130.4 ± 14.0*	7.7 ± 3.3*	199.0 ± 50.0*	27.5 ± 3.8*
Cd + $(PhSe)_2$	110.8 ± 12.9#	7.2 ± 1.2*	152.0 ± 94.8#	23.4 ± 5.8*

Data are mean±S.D. from six animals in each group. (*) Denoted $p<0.05$ as compared to control group (one-way ANOVA/Duncan). (#) Denoted $p<0.05$ as compared to Cd group.

Table 3 - Effect of subchronic treatment with cadmium and $(PhSe)_2$ therapy on ascorbic acid levels in mice liver, kidney, brain and spleen

Ascorbic acid levels (μmol/g wet tissue)				
Group	Liver	Kidney	Brain	Spleen
Control	266.8 ± 25.5	172.8 ± 2.2	410.4 ± 49.9	433.6 ± 11.0
$(PhSe)_2$	278.6 ± 10.0	140.6 ± 13.6*	397.2 ± 19.4	435.4 ± 40.4
Cd	287.5 ± 37.9	153.8 ± 13.5*	404.6 ± 32.4	386.9 ± 30.9*
Cd + $(PhSe)_2$	284.4 ± 28.1	179.8 ± 24.7#	406.5 ± 43.4	377.1 ± 16.2*

Data are mean±S.D. from six animals in each group. (*) Denoted $p<0.05$ as compared to control group (one-way ANOVA/Duncan). (#) Denoted $p<0.05$ as compared to Cd group.

Table 4 - Effect of subchronic treatment with cadmium and $(PhSe)_2$ therapy on δ-ALA-D activity in mice liver, kidney, brain and spleen

δ-ALA-D activity (nmol PBG/mg protein/h)				
Group	Liver	Kidney	Brain	Spleen
Control	12.8 ± 0.3	6.6 ± 0.3	3.8 ± 0.9	2.6 ± 0.1
$(PhSe)_2$	12.4 ± 0.3	6.9 ± 0.3	3.9 ± 0.6	3.2 ± 0.4
Cd	9.7 ± 0.7*	4.4 ± 0.2*	3.8 ± 0.8	0.7 ± 0.2*
Cd + $(PhSe)_2$	12.5 ± 1.3#	6.3 ± 0.1#	3.6 ± 0.9	2.1 ± 0.2#

Data are mean±S.D. from six animals in each group. (*) Denoted $p<0.05$ as compared to control group (one-way ANOVA/Duncan). (#) Denoted $p<0.05$ as compared to Cd group.

Table 5 - Effect of subchronic treatment with cadmium and $(PhSe)_2$ therapy on LDH, AST, ALT activities and creatinine, urea levels in mice

Group	LDH (IU/L)	AST (IU/L)	ALT (IU/L)	Creatinine (mg/dL)	Urea (mg/dL)
Control	370.4 ± 77.4	271.4 ± 63.4	30.4 ± 9.3	0.2 ± 0.05	29.4 ± 5.0
$(PhSe)_2$	409.0 ± 54.6	314.4 ± 114.9	38.6 ± 7.4	0.2 ± 0.05	28.6 ± 3.8
Cd	736.3 ± 84.5*	522.6 ± 108.0*	129.0 ± 54.9*	0.3 ± 0.07	35.8 ± 7.4
Cd + $(PhSe)_2$	643.0 ± 54.0*	347.8 ± 122.9	90.3 ± 18.0*	0.25 ± 0.06	25.5 ± 5.5

Data are mean±S.D. from six animals in each group. (*) Denoted $p<0.05$ as compared to control group (one-way ANOVA/Duncan).

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5– DISCUSSÃO

A terapia com agentes quelantes é o modo mais efetivo de tratar a intoxicação por metais tóxicos, tais como o arsênio, cádmio, chumbo e mercúrio. Porém, o tratamento prolongado com um agente quelante pode provocar distúrbios hematopoiéticos (Flora e Kumar, 1993), alterações no metabolismo celular, desequilíbrio na síntese de DNA, RNA e proteínas (Fischer et al., 1975) ou desequilíbrio de elementos traços (Cantilena e Klaassen, 1982). Pode-se inferir então que, embora os agentes quelantes sulfidrílicos sejam efetivos em remover os metais tóxicos nas intoxicações, eles apresentam alguns efeitos tóxicos *per se*. De fato, trabalhos prévios do nosso grupo demonstraram que os agentes quelantes ditiólicos, entre eles o BAL, apresentam atividade inibitória *per se* sobre a enzima δ-aminolevulinato desidratase (δ-ALA-D) de rim (Emanuelli et al., 1996), enquanto o DMPS e o DMSA apresentam efeito inibitório sobre a enzima hepática (Nogueira et al., 2003b), ambos em camundongos.

O presente trabalho **(Artigo 1)** demonstrou claramente que o BAL, o DMPS e o DMSA inibem a atividade da enzima δ-ALA-D de eritrócitos humanos *per se*. A importância destes achados está no fato de que a inibição da δ-ALA-D pode levar ao acúmulo do seu substrato, o ALA (Emanuelli et al., 2001), o qual pode se auto-oxidar, dando origem às espécies reativas de oxigênio. Estas, por sua vez, são nocivas aos sistemas biológicos, uma vez que podem oxidar biomoléculas importantes, provocando injúria tecidual e morte celular (Yu,1994).

Uma vez que a atividade da δ-ALA-D é altamente sensível à presença de compostos capazes de oxidar seus grupos sulfidrílicos, como os metais (Rocha et al., 1995, Rodrigues et al., 1996), e também é sensível a compostos capazes de remover os íons zinco de sua estrutura (Jaffe, 1995; Beber et al., 1998; Emanuelli et al., 1998), uma maneira de se estudar o mecanismo de inibição enzimática pelos quelantes seria utilizar compostos redutores tiólicos e cloreto de zinco.

O agente redutor ditiol DTT, mas não o monotiol cisteína, protegeu e reverteu a inibição enzimática causada por estes compostos. O $ZnCl_2$ também foi efetivo em reduzir os efeitos inibitórios dos agentes quelantes. Outros autores demonstraram que compostos

capazes de oxidar os grupos –SH de enzimas sulfidrílicas poderiam ter sua ação revertida por ditióis e não por monotióis, sugerindo que os compostos em questão poderiam estar se ligando nos dois grupos sulfidrila vicinais da enzima (Laden e Porter, 2001; Gupta e Porter, 2001).

Sendo assim, os resultados **(Artigo 1)** sugerem que os quelantes ditiólicos inibem a atividade da δ-ALA-D de sangue humano por interagir com os grupos –SH vicinais da enzima e também por remover os íons zinco, os quais são essenciais para a atividade desta.

Uma vez que as observações *in vitro* sobre a toxicidade dos agentes quelantes fornecem apenas uma orientação superficial, buscou-se aprofundar os estudos com relação ao potencial tóxico dos agentes quelantes estudados. Estudou-se então, o efeito de um tratamento agudo com estes compostos sobre alguns parâmetros toxicológicos em camundongos **(Artigo 2)**.

Demonstrou-se que o BAL e o DMPS são tóxicos quando administrados agudamente, considerando o efeito inibitório sobre a δ-ALA-D e o aumento nos níveis de TBARS no rim de camundongos. Como considerado anteriormente, esta inibição da δ-ALA-D poderia ser prejudicial e, em associação com o aumento na peroxidação lipídica poderia potencializar a geração de radicais livres. De acordo com essas considerações, evidências recentes do nosso e de outros laboratórios demonstraram que a redução da atividade da enzima está associada com um aumento no estresse oxidativo em roedores (Flora et al., 2002, Soares et al., 2003). Além disso, a inibição da δ-ALA-D renal está de acordo com estudos prévios realizados com camundongos *ex vivo* (Emanuelli et al., 1996).

O DMPS aumentou o nível de zinco no rim e isto poderia estar associado com a mobilização deste metal de outros órgãos para o rim. Considerando que o mecanismo terapêutico dos quelantes envolve a excreção dos metais tóxicos do corpo, uma interação entre estes e os metais endógenos, como o zinco, é provável (Cantilena e Klaassen, 1981). Por outro lado, o BAL causou uma redução nos níveis de zinco hepático, enquanto a atividade da enzima neste tecido foi estimulada. É importante salientar que mesmo que esta redução na concentração deste metal essencial tenha sido significativa, este valor ainda está nos limites toleráveis de zinco no tecido hepático. Esse resultado é importante, pois dados da literatura demonstraram que o EDTA e o BAL inibem a δ-ALA-D *in vitro* por remover o

zinco dos sítios envolvidos em manter os resíduos cisteinil reduzidos (Emanuelli et al., 1998).

As diferenças entre os efeitos dos quelantes observadas neste estudo **(Artigo 2)**, assim como a variação na atividade da δ-ALA-D entre os tecidos (o aumento no sangue, fígado, cérebro e a diminuição no rim) podem estar relacionadas com as propriedades químicas e físicas dos mesmos. Neste contexto, pode-se sugerir que o DMSA produziu poucas alterações bioquímicas, quando comparado ao BAL e ao DMPS. Entretanto, o mecanismo exato da toxicidade induzida pelos quelantes não está claro, mas poderia envolver uma variedade de alvos moleculares ou celulares.

A exposição das populações humanas a uma variedade de metais tóxicos é um problema de saúde pública (Goyer, 1996). O cádmio é um dos elementos não-essenciais mais abundantes, devido a sua grande utilização na indústria (Page et al., 1986). O alto risco de intoxicação por cádmio pelo homem e a toxicidade das exposições agudas e crônicas são bem descritos (Fowler, 1991; Goyer, 1996). Muitas evidências sugerem que o aumento da peroxidação lipídica seria o processo primário responsável pela ação tóxica do cádmio (Manca et al., 1991). Sendo assim, acredita-se que a utilização de antioxidantes, sozinhos ou em associação com os agentes quelantes, seria uma alternativa mais eficaz para o tratamento das intoxicações por cádmio (Flora, 1999; Pande et al., 2001; Tandon et al., 2003).

A administração aguda de cádmio causou um aumento na peroxidação lipídica, uma redução no conteúdo de ácido ascórbico, um aumento na concentração de hemoglobina, e uma inibição da atividade da δ-ALA-D e da SOD em testículo de camundongos **(Artigo 3)**. Neste trabalho, pela primeira vez, demonstrou-se que a exposição ao cádmio altera a atividade da δ-ALA-D testicular e, com relação a este parâmetro de toxicidade, o $(PhSe)_2$ e o DMSA foram efetivos em reverter a atividade enzimática aos níveis do controle. Estes compostos foram também efetivos em restaurar o aumento nos níveis de TBARS em testículo, sendo que o $(PhSe)_2$ demonstrou ser mais eficaz do que o DMSA quando doses mais elevadas de cádmio foram utilizadas. Nosso grupo demonstrou anteriormente que o $(PhSe)_2$ é um potente antioxidante *in vitro* (Rossato et al., 2002) e apresenta atividade tiol-peroxidase maior do que outros disselenetos e que o ebselen (Meotti et al., 2004).

A terapia com $(PhSe)_2$ e DMSA, sozinhos ou associados, não foi efetiva em recuperar as defesas antioxidantes no testículo, uma enzimática (SOD) e outra não-enzimática (ácido

ascórbico), alteradas pela exposição ao cádmio. De maneira geral, a utilização da terapia combinada ($(PhSe)_2$ e DMSA) não foi melhor do que a monoterapia em restaurar os parâmetros toxicológicos avaliados neste modelo de dano testicular induzido pelo cádmio **(Artigo 3)**. Entretanto, o uso do $(PhSe)_2$ como antioxidante parece ser útil em melhorar a peroxidação lipídica, a qual é considerada um processo importante para a toxicidade do cádmio.

Ao contrário da terapia com DMSA, a terapia utilizando o DMPS não foi eficaz em reverter o aumento dos níveis de TBARS induzido pelo cádmio em testículo de camundongos, causando somente uma pequena redução destes **(Artigo 4)**. Por outro lado, o $(PhSe)_2$, sozinho ou em associação com o DMPS foi efetivo em reverter este parâmetro. Estes compostos, $(PhSe)_2$ e DMPS, foram eficientes em melhorar a inibição da δ-ALA-D testicular induzida pelo cádmio. Além disso, tanto o $(PhSe)_2$ quanto o DMPS foram capazes de reduzir o acúmulo de cádmio nos testículos, sendo que a terapia concomitante com estes compostos mostrou-se mais eficaz.

A exposição ao cádmio causou uma elevação nas atividades das enzimas ALT e AST no plasma de camundongos, o que poderia indicar uma redução da atividade destas enzimas no fígado, decorrentes de um dano neste tecido **(Artigo 4)**. De fato, Rana et al. (1996) e El-Demerdash et al. (2004) demonstraram que o cádmio causou alterações nas transaminases de rato. Portanto, uma elevação destas enzimas no plasma poderia ser decorrente da liberação destas do citosol hepático para a circulação sangüínea (Navarro et al., 1993), o que poderia indicar um efeito hepatotóxico do cádmio. A terapia com DMPS e $(PhSe)_2$ não foi eficaz em restaurar o aumento da atividade da ALT e da AST induzido pelo cádmio, com exceção da terapia concomitante (DMPS e $(PhSe)_2$), a qual foi efetiva em melhorar o aumento da AST causado pela maior dose de cádmio testada (5 mg/kg). Uma vez que o fígado exerce um papel central na detoxificação de xenobióticos, o fato de os compostos utilizados na terapia não alterarem a atividade da AST e da ALT *per se*, sugere que estes compostos não induzem dano hepático.

Em resumo, os resultados indicam que a terapia combinada ($(PhSe)_2$ e DMPS) foi melhor do que a monoterapia em reduzir os níveis de cádmio nos testículos e em restaurar os níveis de AST no plasma dos animais expostos ao cádmio **(Artigo 4)**. Por outro lado, a

terapia combinada não promoveu melhora dos outros parâmetros avaliados neste modelo experimental.

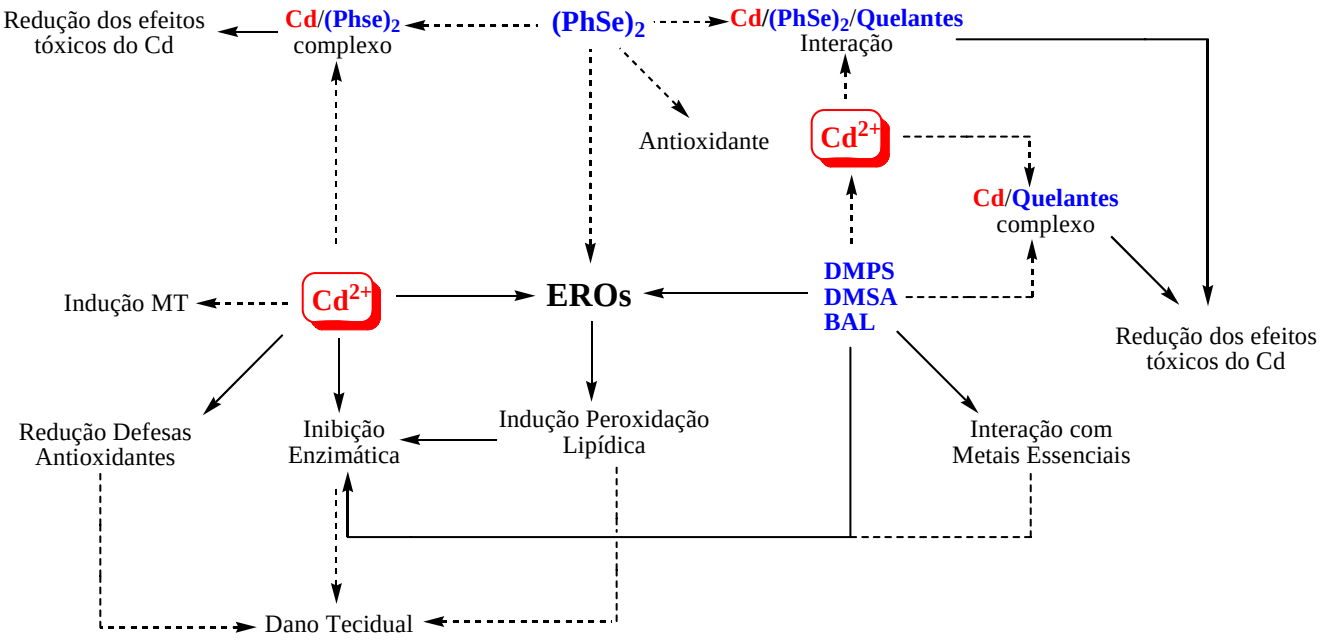
A exposição sub-crônica ao cádmio promoveu um aumento na peroxidação lipídica de tecidos de camundongos. Até onde sabemos, este é o primeiro relato demonstrando que o $(PhSe)_2$ apresenta atividade antioxidante em tecidos de camundongos após uma intoxicação sub-crônica por cádmio, uma vez que este composto foi capaz de reduzir o aumento dos níveis de TBARS no fígado e no cérebro de camundongos por este metal **(Artigo 5)**.

Os efeitos desta exposição parecem ser mais prejudiciais ao tecido hepático do que ao tecido renal, uma vez que, além do aumento nos níveis de TBARS hepático, observou-se uma inibição da atividade da δ-ALA-D, bem como uma elevação das enzimas ALT e AST plasmáticas. Ao contrário, no rim observou-se uma redução na peroxidação lipídica e não houve alteração nos níveis de uréia e creatinina plasmáticos **(Artigo 5)**. Isto pode ser explicado pelo fato de que o cádmio é depositado primeiramente no fígado, onde ele induz e se liga a metalotioneínas (MT) (Shaikh and Lucis, 1972). Com o tempo, o complexo CdMT é lentamente liberado para a circulação e, subsequentemente a proteína é filtrada do sangue pelos glomérulos renais, onde este complexo é degradado (Tohyama et al., 1981). Então, os íons cádmio liberados da degradação do complexo CdMT se ligam a metalotioneínas renais pré-existentes e também a metalotioneínas novamente induzidas (sintetizadas). Acredita-se que somente o excesso de cádmio não ligado às MT é capaz de causar nefrotoxicidade (Goyer et al., 1989), possivelmente pela geração de radicais livres. Sendo assim, entende-se que na intoxicação por cádmio, primeiro ocorre a deposição deste metal no fígado, podendo causar efeitos tóxicos e, só posteriormente ele poderá causar uma injúria renal.

Sendo assim, pode-se sugerir que a utilização do $(PhSe)_2$ na terapia da intoxicação sub-crônica por cádmio parece ser relevante, uma vez que o mesmo é capaz de reduzir muitos dos efeitos danosos deste metal.

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

Esquema 1 – Visão geral dos efeitos do cádmio, do $(PhSe)_2$ e dos agentes quelantes, 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.

6- CONCLUSÕES

De acordo com os resultados apresentados nesta tese podemos inferir o seguinte:

- Os agentes quelantes ditiólicos BAL, DMPS e DMSA apresentam um efeito inibitório *per se* sobre a atividade da enzima δ-aminolevulinato desidratase de sangue humano *in vitro*. Investigando o possível mecanismo de inibição enzimática por estes compostos, verificou-se que tanto a oxidação dos grupos –SH quanto a remoção dos íons zinco presentes na estrutura da δ-ALA-D estariam envolvidos, em maior ou menor grau, dependendo do composto em questão.

- A administração aguda dos agentes quelantes BAL, DMPS e DMSA causa efeito *per se* sobre alguns parâmetros toxicológicos em camundongos. O BAL e o DMPS são tóxicos quando administrados agudamente, considerando o efeito inibitório destes compostos sobre a δ-ALA-D e o aumento nos níveis de TBARS no rim destes animais. O DMSA parece ser o menos tóxico dos compostos testados, já que produziu poucas alterações bioquímicas.

- A exposição aguda ao cádmio causa dano testicular associado ao aumento da peroxidação lipídica e a alterações nas defesas antioxidantes. O DMPS e o DMSA foram efetivos em melhorar alguns parâmetros alterados pelo cádmio. A utilização do $(PhSe)_2$ como antioxidante na terapia da indução de dano testicular pelo cádmio demonstrou ser eficaz, tanto sozinho quanto associado aos quelantes.

- A exposição sub-crônica ao cádmio causa alterações importantes em tecidos de camundongos. O $(PhSe)_2$ foi efetivo em reverter vários dos parâmetros alterados pela exposição sub-crônica ao cádmio em tecidos de camundongos. Dessa forma, pode-se considerar este composto como uma droga potencial no tratamento de intoxicações por cádmio e, portanto, acentuar os estudos com relação a este composto a fim de tornar a sua utilização terapêutica viável.

7- PERSPECTIVAS

Tendo em vista os bons resultados obtidos na terapia de intoxicações por cádmio com a utilização dos quelantes e com o $(PhSe)_2$, poderíamos aprofundar ainda mais estes estudos com relação ao mecanismo de ação destes compostos. Ainda, poderíamos testar outros compostos orgânicos de selênio com atividade antioxidante potencial, a fim de descobrir novas terapias que auxiliem na intoxicação por cádmio. Dessa forma, poderíamos realizar este estudo a partir da concretização dos seguintes objetivos:

- Identificar, detalhadamente, o mecanismo pelo qual o DMPS, o DMSA e o $(PhSe)_2$ são capazes de melhorar os parâmetros toxicológicos alterados pela intoxicação por cádmio.
- Demonstrar, a partir de estudos mais detalhados, o mecanismo pelo qual o $(PhSe)_2$ é capaz de reduzir a concentração de cádmio tecidual.
- Estudar outros compostos orgânicos de selênio com atividade antioxidante potencial no modelo de dano testicular induzido por cádmio em camundongos, sozinhos e em associação com o DMPS e o DMSA.
- Testar um possível efeito benéfico destes mesmos compostos em modelos de intoxicação sub-crônica e crônica por cádmio, avaliando, além das alterações bioquímicas, possíveis alterações histopatológicas em tecidos de camundongos.

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