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Tese de Doutorado

EFEITOS DA ADMINISTRAÇÃO DO DISSELENETO DE DIFENILA SOBRE O DANO HEPÁTICO INDUZIDO POR 2-NITROPROPANO, CÁDMIO E TETRACLORETO DE CARBONO

Lysandro Pinto Borges

Santa Maria, RS, Brasil

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por

Lysandro Pinto Borges

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

2008

Universidade Federal de Santa Maria
Centro de Ciências Naturais e Exatas
Programa de Pós-Graduação em Bioquímica Toxicológica

A Comissão Examinadora, abaixo assinada, aprova a Tese de Doutorado

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Elaborada por **Lysandro Pinto Borges** como requisito parcial para a obtenção do grau de **Doutor em Bioquímica Toxicológica**

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Prof. Dr. Bernardo Baldisserotto

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Prof. Dr. Alexandre Mazzanti

Santa Maria, fevereiro de 2008.

"O mundo é como um espelho

que devolve a cada pessoa o reflexo

de seus próprios pensamentos.

A maneira como você encara a vida

é que faz toda diferença."

Luís Fernando Veríssimo.

AGRADECIMENTOS

Agradeço, primeiramente, a Deus que me deu o dom da vida.

Agradeço à minha família, especialmente aos meus avós (Eda e Chiquinho), que ajudaram na minha criação. Por todo incentivo, apoio e amor que sempre tiveram comigo. Vocês foram meus maiores incentivadores e nunca mediram esforços para me ajudar no que foi preciso. Eu amo vocês!

A minha mãe e tia, obrigado pelo seu amor, amizade, carinho e incentivo. Amo muito vocês. Ao meu pai pelo apoio financeiro e orações, “sei que quando não estavas presente em alguns momentos de minha vida era por estar cuidando de meu futuro”, obrigado por tudo, amo você.

À Sessa, mulher, companheira, colega de curso, amiga de todas as horas e meu grande amor, por dividir comigo minhas angústias, alegrias, por todo seu apoio e amor. Obrigado por estar sempre ao meu lado em todos os momentos e por ser a pessoa tão especial que és. Admiro-te e a amo muito!

Profa. Cristina, um simples “obrigado” é pouco para expressar toda gratidão que eu tenho por você, agradeço por ter me aceitado no seu grupo e por ter acreditado em mim. Na vida, o destino coloca pessoas em nossos caminhos, das quais levamos algo de bom e deixamos alguma coisa. Espero ter deixado contigo o meu melhor. Obrigado por tudo!

Ao GZ, meu orientador, por ter me aceitado no seu grupo. Ao pessoal do meu laboratório, pela amizade e companheirismo: Diego “Schumaker”, Panatieri, Jesus, Angélica, Joel, Ricardo, Patrícia, Flávia, Dani, Carol, Cirilo e Benhur.

Ao professor João, pelos ensinamentos, pela amizade e por sua colaboração no meu trabalho. Tua humildade, capacidade e prestatividade, ficarão para sempre na minha lembrança e também ao pessoal do teu laboratório.

A Aninha e ao Marlon, por terem me ajudado a concretizar este trabalho e por serem pessoas muito especiais para mim. Muito obrigado!

A todo o pessoal do laboratório: em especial ao Ricardo (meu parceiro em vários trabalhos), quero ser teu eterno amigo, valeu pela amizade e parceria. Ao Alexandre e Simone Weis, exemplos de dedicação e amor ao que fazem, continuem sendo assim. A Lucielli, Nutri, Gabi, Cristiano, Lio, Ethel e a todos os outros colegas que direta ou indiretamente me auxiliaram, obrigado pela amizade, pela colaboração, pelas festas, pela alegria; é muito bom fazer parte deste grupo. Adoro todos vocês!

Ao Sérgio (Histologia) e ao Prof. Figuera (Patologia Veterinária) obrigado pela amizade, ensinamentos e excelência em seu trabalho, que ajudou a concretizar minhas idéias. Obrigado, de coração!

A todos os professores do Programa de Pós-Graduação em Bioquímica Toxicológica pela atenção que sempre tiveram comigo.

À Angélica por ter cuidado da parte burocrática sempre com bom humor e amizade e ao Rinaldo por ter tomado conta dos ”animais de laboratório” e pela sua amizade, sem o teu trabalho, o nosso seria impossível. Valeu amigo!

A todos, que de alguma forma colaboraram para a realização deste trabalho, o meu “muito obrigado”!

RESUMO

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

EFEITOS DA ADMINISTRAÇÃO DO DISSELENETO DE DIFENILA SOBRE O DANO HEPÁTICO INDUZIDO POR 2-NITROPROPANO, CÁDMIO E TETRACLORETO DE CARBONO

AUTOR: Lysandro Pinto Borges
ORIENTADOR: Gilson Rogério Zeni
CO-ORIENTADOR: Cristina Wayne Nogueira
DATA E LOCAL DA DEFESA: Santa Maria, fevereiro de 2008.

O fígado apresenta extraordinária pluralidade funcional, destacando-se no controle de produção de energia, defesa imunológica e reserva volêmica. No meio ambiente e ocupacionalmente, o ser humano está exposto a uma variedade de compostos hepatotóxicos, como por exemplo, no uso de tintas e seus derivados (2-nitropropano), reagentes químicos (tetracloreto de carbono) e na exposição ao cigarro (cádmio e 2-nitropropano). Portanto, é interessante o estudo de terapias que previnam ou até mesmo revertam à intoxicação causada por estes compostos. Considerando o crescente interesse por compostos orgânicos de selênio, em especial o disseleneto de difenila ($(PhSe)_2$) que possui propriedades farmacológicas mais amplas como: efeitos anti-úlcera, antiinflamatório e antinociceptivo, anti-hiperglicemiante, protege contra a discinesia orofacial induzida por reserpina e haloperidol e pode atuar na facilitação da formação de memória em camundongos. Deste modo, os efeitos hepatoprotetores deste composto frente a diferentes modelos de dano hepático (2-nitropropano, cádmio e tetracloreto de carbono) foram examinados. Os resultados obtidos neste estudo demonstraram que a administração de $(PhSe)_2$ (100 μmol/kg) reduziu os níveis de marcadores hepáticos e os níveis de peroxidação lipídica quando comparado ao grupo tratado com 2-nitropropano (2-NP). Além disso, os exames histológicos revelaram que o tratamento com 2-NP causou alterações degenerativas nos hepatócitos e que o $(PhSe)_2$ foi capaz de proteger, evidenciando o efeito hepatoprotetor desse composto sobre o dano hepático induzido por 2-NP. O efeito do pós-tratamento com $(PhSe)_2$ sobre o dano hepático induzido com 2-NP também foi investigado. Este composto restaurou a atividade plasmática das enzimas aminotransferases e os níveis de uréia quando comparado ao grupo tratado com 2-NP. Na maior dose (100 μmol/kg), o $(PhSe)_2$ causou uma diminuição na atividade da enzima γ-glutamil transferase (GGT) e restituiu o aumento nos níveis de peroxidação lipídica hepáticos e renais quando comparado ao grupo tratado com 2-NP. O tratamento com 2-NP reduziu a atividade hepática da catalase, entretanto não alterou a atividade da superóxido dismutase (SOD) e os níveis de ácido ascórbico, sugerindo que a inibição da CAT pode estar relacionada com o aumento nos níveis de peroxidação lipídica hepática nos ratos tratados com 2-NP. Resultados similares foram encontrados quando o dano hepático foi induzido por cádmio (Cd), um

contaminante ambiental implicado em várias doenças. O conteúdo de Cd determinado nos ratos expostos ao cloreto de cádmio ($CdCl_2$) provêm evidências de que o fígado é o maior alvo da toxicidade deste metal. A concentração de cádmio no fígado foi em torno de 3 vezes maiores que os níveis encontrados no rim. O $(PhSe)_2$ reduziu em torno de 6 vezes os níveis deste metal no fígado dos ratos expostos ao $CdCl_2$. Além disso, a administração de $(PhSe)_2$ causou uma redução nos níveis de malondialdeído plasmáticos (MDA), na atividade das aminotransferases, na fosfatase alcalina (ALP), lactato desidrogenase (LDH) e GGT quando comparado ao grupo tratado com cádmio. Em conclusão, esse estudo demonstrou que o tratamento concomitante com $(PhSe)_2$ reduziu a hepatotoxicidade e o dano celular em fígado de ratos expostos ao cádmio. O mecanismo proposto para ação do $(PhSe)_2$ pode ser devido as suas propriedades antioxidantes ou pela sua capacidade de formar um complexo com Cd. Em contraste, a administração de $(PhSe)_2$ potencializou o dano induzido por tetracloreto de carbono (CCl_4), o que foi demonstrado pelo aumento dos níveis de marcadores bioquímicos (AST, ALT, ALP, GGT and BT) e pela severa alteração na histologia. Esses estudos também demonstraram que a administração de $(PhSe)_2$ potencializou os níveis de peroxidação lipídica com consequente depleção das defesas antioxidantes, como a catalase e o ácido ascórbico, sugerindo que o dano oxidativo está relacionado com este efeito. Considerando os resultados obtidos, podemos sugerir que o disseleneto de difenila apresenta um efeito hepatoprotetor dependendo do modelo experimental.

Palavras-chave: dano hepático, selênio, disseleneto de difenila, tetracloreto de carbono, cádmio, 2-nitropropano.

ABSTRACT

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

EFFECTS OF DIPHENYL DISELENIDE ADMINISTRATION ON LIVER DAMAGE INDUCED BY 2-NITROPROPANE, CADMIUM AND CARBON TETRACHLORIDE

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CO-ADVISOR: Cristina Wayne Nogueira
DATE AND PLACE OF THE DEFENSE: Santa Maria, 2008

The liver presented exceptional characteristics, like controlling energy production, immunological defenses, and blood reserve. In the environment like in the work place, the human is exposed to a different kind of hepatotoxic compounds, for example, on inks and derivatives (2-nitropropane), chemical reagents (carbon tetrachloride) and in tobacco smoke (2-nitropropane and cadmium). In fact, is interesting studies of therapies which protect or ameliorated the damage induced by these compounds. Considering the growing interesting around organochalcogens, in special interest, diphenyl diselenide $(PhSe)_2$, which posses important pharmacological properties, such as: anti-ulcer, antiinflammatory, antinociceptive, anti-hyperglycemic, protected against orofacial diskinesia induced by reserpine and halopheridol and may act on memory facilitation in mice, the hepatoprotective properties of this compound induced by different models of liver damage (2-nitropropane, cadmium and carbon tetrachloride) were examined. The results demonstrated that $(PhSe)_2$ (100 μmol/kg) significantly reduced hepatic markers levels when compared to 2-nitropropane (2-NP) group. Treatment with diphenyl diselenide, at all doses, effectively protects against the increase of lipid peroxidation when compared to 2-NP group. In addition, histological examination revealed that 2-NP treatment causes a moderate swelling and degenerative alterations on hepatocytes and $(PhSe)_2$ protects against these alterations. This study evidences the protective effect of diphenyl diselenide by 2-NP-induced acute hepatic damage. In addition the effect of post-treatment with $(PhSe)_2$ on liver damage induced by 2-NP was also examined. $(PhSe)_2$ effectively restored the increase of aminotransferase activities and urea level when compared to the 2-NP group. At the highest dose (100 μmol/kg), $(PhSe)_2$ decreased γ-glutamyl transferase activity (GGT) and ameliorated the increase of hepatic and renal lipid peroxidation when compared to 2-NP group. 2-NP reduced catalase activity (CAT) and did not alter superoxide dismutase activity (SOD) nor ascorbic acid level. This study points out the involvement of CAT activity in 2-NP-induced acute liver damage and suggests that the post-treatment with diphenyl diselenide was effective in restoring the hepatic damage induced by 2-NP. Similar results were obtained with cadmium (Cd), an environmental toxic metal implicated in human diseases. Cadmium content determined in the tissue of rats exposed to cadmium chloride ($CdCl_2$) provides evidence that the liver is the major cadmium target. The concentration of cadmium in liver was about three fold higher than that in kidney, and $(PhSe)_2$ reduced about

six fold the levels of this metal in liver of rats exposed. Rats exposed to $CdCl_2$ showed histological alterations abolished by $(PhSe)_2$ administration. In addition, $(PhSe)_2$ administration ameliorated plasma malondialdehyde (MDA) levels, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and GGT activities increased by $CdCl_2$ exposure. In conclusion, this study demonstrated that co-treatment with $(PhSe)_2$ ameliorated hepatotoxicity and cellular damage in rat liver after sub-chronic exposure with $CdCl_2$. The proposed mechanisms by which $(PhSe)_2$ acts in this experimental protocol are its antioxidant properties and its capacity to form a complex with Cd. On the contrary, the administration of $(PhSe)_2$ potentiated acute hepatic damage induced by carbon tetrachloride (CCl_4), as manifested by an increase in biochemical parameters (AST, ALT, ALP, GGT and BT) and severe alteration in histopathology. This study also demonstrated a potentiation of lipid peroxidation levels and a consequent depletion of important antioxidant defenses including catalase and ascorbic acid, suggesting that the oxidative damage is related to the potentiation effect induced by $(PhSe)_2$. Considering the results obtained, could be suggested that $(PhSe)_2$ present a hepatoprotective effect depending of experimental protocol.

Key words: liver damage, selenium, diphenyl diselenide, carbon tetrachloride, cadmium, 2-Nitropropane.

LISTA DE FIGURAS

Revisão Bibliográfica

Artigo 1

Artigo 2

LISTA DE TABELAS

LISTA DE ESQUEMAS

LISTA DE ABREVIATURAS

AFP	α-feto proteína
ALT/TGP	alanina aminotransferase
ANOVA	análise de variância
AST/TGO	aspartato aminotransferase
ATP	adenosina trifosfato
BT	bilirrubina total
BD	bilirrubina direta
CAT	catalase
Cd	cádmio
$CdCl_2$	cloreto de cádmio
CYP	sistema P-450
DI_{50}	dose que inibe a resposta em 50%
DL_{50}	dose que causa 50% de morte
EROs	espécies reativas de oxigênio
FAL	fosfatase alcalina
GGT	γ- glutamil transferase
GPx	glutationa peroxidase
GSH	glutationa reduzida
HAS	ácido orto-sulfônico hidroxilamina
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
IPHA	N-isopropil hidroxilamina
MDA	malondialdeído
MT	metalotioneína
NAC	N-acetilcisteína
NO	óxido nítrico
NOS	óxido nítrico sintase
p.o.	per via oral
PhSeSePh	disseleneto de difenila
$(PhSe)_2$	disseleneto de difenila
Se	selênio
SOD	superóxido dismutase
s.c.	subcutânea
SNC	sistema nervoso central
TBARS	espécies reativas ao ácido tiobarbitúrico

SUMÁRIO

APRESENTAÇÃO

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

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

No item **PERSPECTIVAS** estão expostos os possíveis estudos para continuação do estudo do autor, referente a esse assunto.

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

1- INTRODUÇÃO

O fígado apresenta extraordinária pluralidade funcional, destacando-se no controle de produção de energia, defesa imunológica e reserva volêmica (Kalil e col., 2001). Sua importância no metabolismo e armazenamento de vitaminas, carboidratos, proteínas e lipídeos, bem como na metabolização e excreção de compostos endógenos e exógenos circulantes, torna complexo o estudo de sua fisiologia. Conseqüentemente, graças à amplitude funcional hepática, este órgão é constantemente exposto a substâncias do meio externo, atuando como órgão alvo de diversos xenobióticos (Motta e col., 2002). Os sinais e sintomas que refletem algum transtorno hepático são: astenia, dor abdominal, náusea, vômito, prurido e icterícia. Além destes sintomas, exames laboratoriais são utilizados para confirmar o diagnóstico de doença hepática e sua severidade, sendo que a insuficiência hepática pode levar a falência de outros órgãos como o encéfalo, rins e coração (Kim e col., 1998).

No meio ambiente e ocupacionalmente, o ser humano está exposto a uma variedade de compostos hepatotóxicos, como por exemplo, no uso de tintas e seus derivados (2-nitropropano), reagentes químicos (tetracloreto de carbono) e na exposição ao cigarro (cádmio e 2-nitropropano). Portanto, é interessante o estudo de terapias que previnam ou até mesmo revertam a toxicidade causada por estes compostos (Henry e col., 1999; Kalil e col., 2001).

Os organocalcogênios são reagentes muito utilizados em laboratórios de química como intermediários em reações de síntese orgânica (Paulmier, 1986; Braga e col., 1996; 1997). Recentemente, em virtude da descoberta de suas propriedades biológicas (Parnham e Graf, 1991; Kanda e col., 1999; Nogueira e col., 2004), os organocalcogênios têm sido alvo de estudos em laboratórios de farmacologia. Conseqüentemente, a possível utilização farmacêutica destes compostos motiva estudos toxicológicos e revela a possibilidade futura de sua utilização no campo da clínica médica.

O disseleneto de difenila, um composto orgânico de selênio, demonstrou propriedades relevantes como: antioxidante (Rossato e col., 2002), antiinflamatórias e antinociceptivas (Nogueira e col., 2003c; Savegnago e col. 2007), anti-úlcera

(Savegnago e col., 2006) e hepatoprotetoras em ratos diabéticos (Barbosa e col., 2006). Baseado nas considerações acima, torna-se importante a avaliação do efeito do disseleneto de difenila frente a modelos experimentais de dano hepático. Além disso, a futura utilização do disseleneto de difenila no tratamento de enfermidades hepáticas motiva ainda mais nossos estudos.

2- REVISÃO BIBLIOGRÁFICA

2.1. Fígado

O fígado é a maior víscera do organismo, pesa em torno de 1200g a 1600g no adulto, ou seja, 2% do peso corpóreo. Localiza-se no quadrante superior direito abdominal, apresentando abundante suprimento sanguíneo proveniente de dois vasos: a artéria hepática e a veia portal. A artéria hepática, uma ramificação da aorta, fornece o sangue com oxigênio ao fígado. A veia portal drena o sangue do sistema digestório (estômago, intestinos, pâncreas e baço) diretamente ao fígado. A importância fisiológica do fluxo sanguíneo portal, é que todas as substâncias provenientes do sistema digestório, com exceção dos lipídeos, passam inicialmente pelo fígado antes de atingir o sistema circulatório. O fígado possui uma estrutura anatômica única. As células hepáticas estão em contato com a circulação sanguínea de um lado e o canalículo biliar de outro. Deste modo, o hepatócito tem uma grande área de contato tanto com um sistema nutriente proveniente dos sinusóides da veia portal e um sistema de escoamento quanto com o canalículo biliar que transporta as secreções e excreções dos hepatócitos (Motta e col., 2002; Kalil e col., 2001).

A fisiologia hepática é altamente especializada no cumprimento de diversas funções conhecidas, tais como: metabólicas, excretoras, secretoras, armazenamento, protetoras, circulatórias e de coagulação sanguínea. Dentre as funções citadas, uma das mais relevantes consiste na função de desintoxicação, através do sistema microssomal de biotransformação de xenobióticos (sistema citocromo P-450 ou CYP). A biotransformação de xenobióticos consiste na conversão de substâncias lipofílicas em

substâncias polares, passíveis de excreção. Esse processo de biotransformação é crucial para eliminação de compostos tóxicos. As principais conseqüências do metabolismo hepático são: a meia-vida biológica é diminuída, a duração da exposição é reduzida e a atividade biológica e sua duração podem ser alteradas. O metabolismo é realizado por enzimas, muitas das quais são específicas e estão localizadas principalmente no retículo endoplasmático. Algumas estão localizadas no citosol e poucas são encontradas em outras organelas como por exemplo, as mitocôndrias. As reações de biotransformação podem ocorrer em duas fases distintas: fase I e II. As reações de fase I são alterações que ocorrem na molécula original através da adição de grupos funcionais, os quais tendem a ser conjugados na fase II. A maioria das reações de biotransformação podem ser divididas em fase I e II, entretanto alguns produtos de fase II podem ser metabolizados em reações de fase III. As principais reacões de fase I são oxidação, redução e hidrólise. A maioria das reações de oxidação é catalisada pelas enzimas monooxigenases encontradas no retículo endoplasmático e conhecidas como enzimas microssomais. As reações de fase II são reações de conjugação que envolvem a adição de grupos endógenos aos xenobióticos, os quais geralmente são polares. Os grupos doados nas reações de conjugação incluem derivados de carboidratos, amino ácidos, glutationa e sulfato. (Timbrell, 1991; Motta e col., 2002).

Esse processo de biotransformação é conhecido como processo de desintoxicação, entretanto em alguns casos podem ser formados metabólitos reativos que são mais tóxicos que os originais. As reações de fase I são as mais comunmente envolvidas nesse processo.

2.2. Dano hepático

As doenças hepáticas são um problema de saúde pública mundial, sendo que a evolução das mesmas inicia-se com a esteatose, hepatite, fibrose, cirrose até o carcinoma hepatocelular (Loguercio e Frederico, 2003; Vitaglione e col., 2004). Evidências crescentes relacionam as espécies reativas de oxigênio com a cascata de eventos que regulam o início e a progressão das doenças hepáticas, independentemente do agente que as originou (Loguercio e Frederico, 2003; Vitaglione e col., 2004). Assim, o uso de terapias antioxidantes (Lima e col., 2007), drogas que

interferem no sistema de metabolização (CYP-450) do agente hepatotóxico e aumentam a atividade de sistemas enzimáticos de defesa são as opções terapêuticas usadas no estudo da hepatotoxicidade (Weber e col., 2003; Porchezhian e col., 2005)

A confirmação do dano hepático é realizada por meio de exames diagnósticos sorológicos específicos, como a dosagem da atividade enzimática das transaminases (AST e ALT), fosfatase alcalina, γ-glutamil-transferase, bilirrubinas e a alfa-fetoproteína (marcador tumoral). São enzimas presentes em vários tecidos, de função intracelular, e sua presença no sangue é conseqüência de liberação anormal para a circulação. A elevação das transaminases no soro pode ocorrer mesmo após agressão celular mínima. A utilização destes marcadores pode delinear o tipo de dano hepático, sua extensão e o prognóstico da doença hepática. Entretanto, somente as técnicas histopatológicas confirmam e complementam a avaliação do dano, auxiliando também na verificação precoce da efetividade de terapias que protejam e/ou revertam a injúria hepática causada por agentes indutores de dano hepático (Henry e col., 1999).

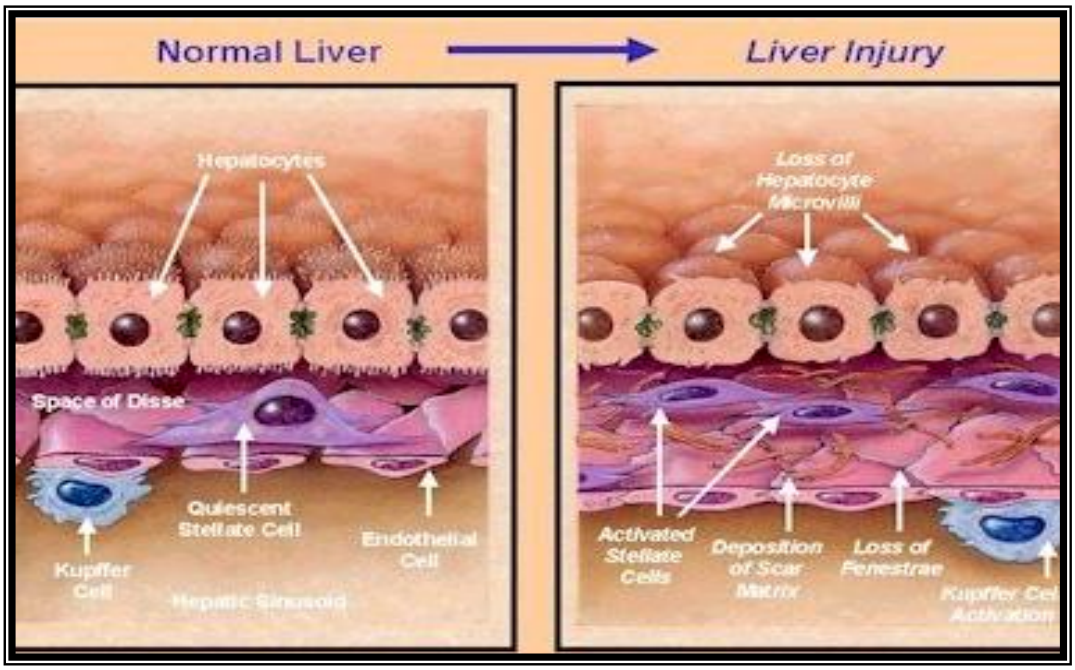

Figura 1: Representação esquemática dos mecanismos da evolução do dano hepático. Space of disse: espaço de Disse; Hepatocytes: hepatócitos; Kupffer cell: célula de Kupffer; Quiescent stellate cell: macrófagos; Endothelial cell: célula endotelial; Hepatic sinusoid: sinusóide hepático; Loss of hepatocyte microvilli: perda das microvisolidades; Activate stellate cells: macrófago ativado; Deposition of scar matrix: depósito de restos celulares; Loss of fenestrae:

perda das fenestras; Kupffer cell activation: célula de Kupffer ativada. Adaptado a partir de Gaw e col., 1999.

2.3. Indutores de dano hepático

2.3.1. 2-Nitropropano (2-NP)

O 2-NP tem sido amplamente utilizado como um intermediário em reações químicas, como solvente, componente de tintas, vernizes, colas, adesivos, no cigarro e em lavanderias nas lavagens a seco (IARC, 1982). Este composto é altamente hepatotóxico (Zitting e col., 1981), hepatocarcinogênico tanto se inalado (Lewis e col., 1979) como por via oral (Fiala e col., 1987), podendo induzir hepatocarcinoma (Petrelli e col., 1993) e linfomas não-Hodkins ou leucemia em humanos expostos ocupacionalmente a solventes que contenham o 2-NP (Roscher e col., 1990; Robbiano e col., 1991).

Após a administração oral (v.o.) ou intraperitonial (i.p.) de 2-NP, este composto muda sua conformação molecular para propano-2-nitronato, com meia-vida de aproximadamente 2 horas, sendo excretado por via pulmonar, renal e fecal (Kohl e col., 1995). O fígado é o orgão alvo da toxicidade do 2-NP, devido ao processo de metabolização pelo sistema P-450 (Ulrich e col., 1978), especificamente pelas isoformas: CYP2B1 e CYP1A2 (Fiala e col., 1987) que favorecem as reações de conjugação mediadas por sulfotransferases e nitroredutases formando os principais metabólitos (N-isopropil hidroxilamina-IPHA e o ácido orto-sulfônico hidroxilamina-HAS) (Figura 2). Estudos demonstraram que esses metabólitos induzem a formação de espécies reativas de oxigênio e nitrogênio (Fiala e col., 1989), 8-amino deoxiguanosina e 8-oxiguanosina (Guo e col., 1990), NOº (Kohl e col., 1995) e malondialdeído (Fiala e col., 1987; 1989).

O 2-NP também demonstrou ser um substrato para a glutationa S-tranferase (Habig e col., 1974), sendo que estes processos de biotransformação que induzem a formação dos metabólitos descritos anteriormente desequilibram a estrutura das membranas celulares causando peroxidação lipídica (Fiala e col., 1989; Zitting e col., 1981), carcinogênese (Roscher e col., 1990), genotoxicidade (Fiala e col., 1989; Kohl e col., 1995), dano ao DNA (Robbiano e col., 1991) e dano pulmonar e renal (Kim e col.,

1998; Guo e col., 1990). As alterações celulares podem ser evidenciadas pela análise histopatológica, a qual demonstra que a administração intraperitoneal de 2-NP induz ao acúmulo de lipídeos no hepatócito, levando a necrose centro-lobular, desgranulação do sistema retículo endotelial e formação de células balonosas, similares às encontradas no tratamento com outros hepatotóxicos (Zitting e col., 1981).

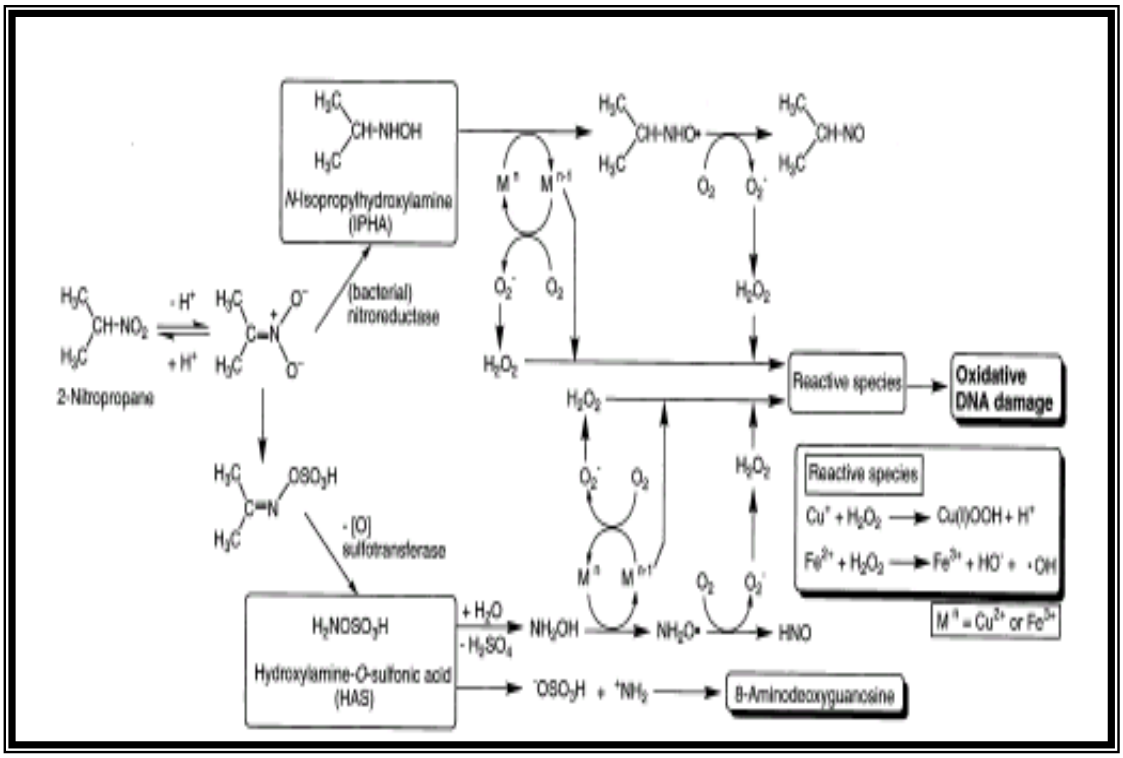

Figura 2: Representação esquemática dos mecanismos de dano oxidativo induzido por 2-NP na presença de metais. Adaptado a partir de Kawanishi e col., 2002.

2.3.2. Cádmio (Cd)

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

A contaminação ambiental com Cd ocorre graças ao amplo uso industrial deste, em processos como produção de plásticos, pigmentos, baterias que contém cádmio e em processos de mineração (Adriano e col., 2001). Esse elemento é absorvido no

organismo em pequenas quantidades, entretanto ele pode se acumular nos tecidos devido a sua longa meia-vida biológica (± 30 anos) (Perry e col., 1962), podendo exercer seu efeito tóxico combinando-se a grupos reativos, como os grupos sulfidrilas (-SH), os quais são essenciais para as funções fisiológicas normais.

O Cd pode afetar vários órgãos como o fígado, rins, pulmões, ovários, ossos, testículos e cérebro (Koizumi e Li, 1992; Santos e col., 2004; 2005).

A intoxicação aguda por Cd produz primariamente injúria hepática e testicular, enquanto a exposição crônica produz dano renal e osteotoxicidade (Rikans e col., 2000). Dessa forma, sob condições de exposição mais prolongada ao Cd, este metal se deposita primariamente no fígado, onde ele induz e se liga às metalotioneínas (MT), podendo também causar efeitos hepatotóxicos. Com o tempo, o complexo Cd-MT hepático é lentamente liberado na circulação (Toyama e Shaikh, 1981) e posteriormente, após filtração glomerular, este complexo é degradado e os íons Cd liberados ligam-se as MT renais pré-existentes ou àquelas recentemente sintetizadas (Cherian, 1978).

De fato, a intoxicação aguda pelo Cd interfere diretamente no metabolismo hepático (Kuester e col., 2002; Zhao e col., 2006), induzindo a formação de radicais livres (Shaikh e col., 1999) (Figura 3), principalmente o ânion superóxido com conseqüente aumento na peroxidação lipídica que culmina com o extensivo dano hepático (Kuester e col., 2002) caracterizado por esteatose, edema, cirrose, fibrose e necrose hepatocelular (Dudley e col., 1982). Além disso, estudos demonstraram que o tratamento com antioxidantes como a glutationa e o alfa-tocoferol reverte o estresse oxidativo induzido pelo Cd (Shaikh e col., 1999), sendo que a terapia com quelantes convencionais não é efetiva na reversão do dano induzido pelo Cd (Jones e Cherian, 1990).

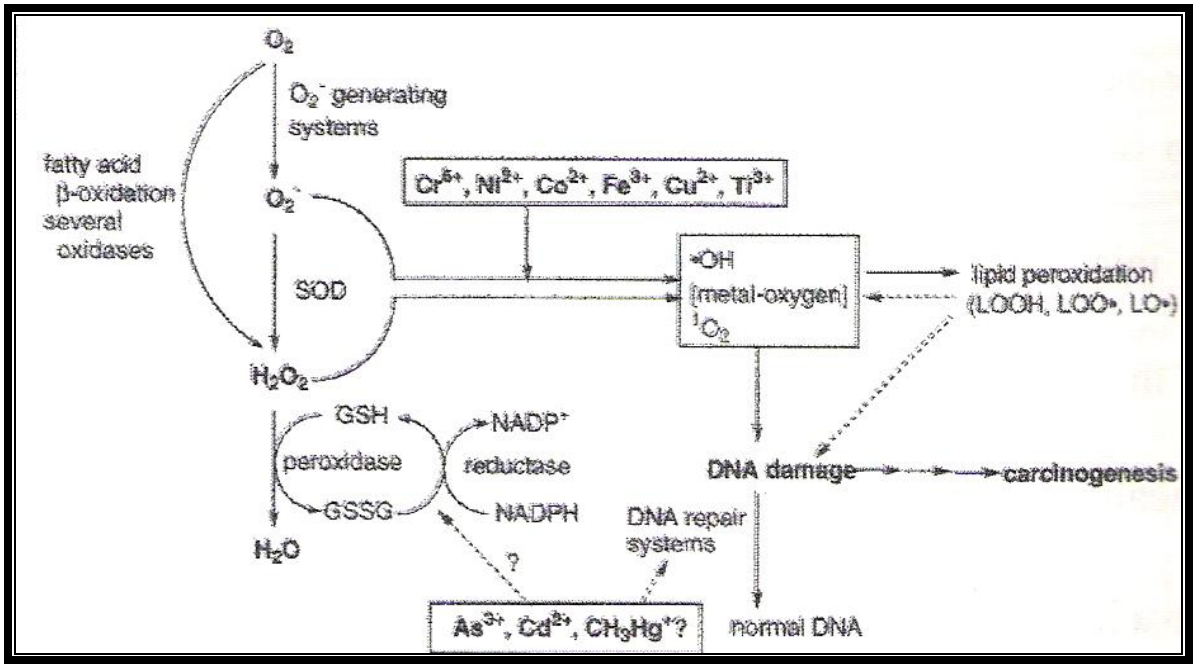

Figura 3: Representação esquemática dos mecanismos de dano oxidativo induzido por espécies reativas de oxigênio na presença de metais. Adaptado a partir de Kawanishi e col., 2002.

2.3.3. Tetracloreto de carbono (CCl_4)

O uso do CCl_4 como intermediário em reações químicas foi severamente restrito, devido a sua severa toxicidade (Weber e col., 2003). Entretanto, a utilização deste composto em protocolos experimentais auxilia a elucidar os mecanismos de hepatotoxicidade e suas conseqüências: inflamação, esteatose, hepatite, fibrose, cirrose e carcinogênese (Lima e col., 2007; Weber e col., 2003; Schatzki, 1963). Além disso, o dano induzido por CCl_4 induz alterações histológicas muito semelhantes às observadas em uma hepatite viral (Weber e col., 2003).

A hepatotoxicidade induzida pelo CCl_4 tem sua gênese em uma reação de desalogenação redutiva catalizada pelo sistema citocromo P-450, sendo que o resultado desta biotransformação é a formação de um radical altamente reativo (CCl_3°) (Recknagel e col., 1967; 1989; 1991; Lima e col., 2007). Este radical reage com o oxigênio para formar o radical triclorometilperoxil (CCl_3OO°)**.** Estes radicais iniciam uma cadeia de reações que direta ou indiretamente interferem em moléculas celulares importantes (ácidos nucléicos, proteínas, lipídeos e carboidratos) desordenando a fisiologia celular, aumentando a peroxidação lipídica (Recknagel e col., 1967; 1989; Lima e col., 2007),

depletando o estoque de glutationa (Recknagel e col., 1989; 1991; Lima e col., 2007) com subseqüente dano e/ou morte celular (Weber e col., 2003; Lima e col., 2007) (Figura 4).

A bioativação do CCl_4 é predominantemente executada pela isoenzima CYP 2E1 (Raucy e col., 1993; Weber e col., 2003), mas em altas doses deste composto, outras isoformas como CYP 2B1, CYP 2B2 e CYP 3A4 são capazes de biotransformar este haloalcano (Weber e col., 2003). Wong e colaboradores (1998) demonstraram em um interessante estudo com camundongos “knockout” para o CYP 2E1, que após a administração do CCl_4 estes animais não desenvolveram dano hepático importante quando comparado ao grupo controle. Estes dados suportam estudos que sugerem que compostos que inibem a CYP 2E1 podem ter ação hepatoprotetora (Kim e col., 1996; Jeong, 1999), enquanto drogas que induzem este sistema isoenzimático podem potencializar o dano hepático induzido por CCl_4 (Weber e col., 2003; Ha e col., 2005).

De fato, o interesse pelo estudo do sistema CYP 2E1 em relação à biotransformação do CCl_4 surgiu quando dados experimentais demonstraram que a localização deste sistema é especificamente na zona centro-lobular hepática (Forkerst e col., 1991), exatamente na mesma região onde o CCl_4 induz as graves alterações histopatológicas como a necrose centrolobular e a degeneração balonosa (Lima e col., 2007).

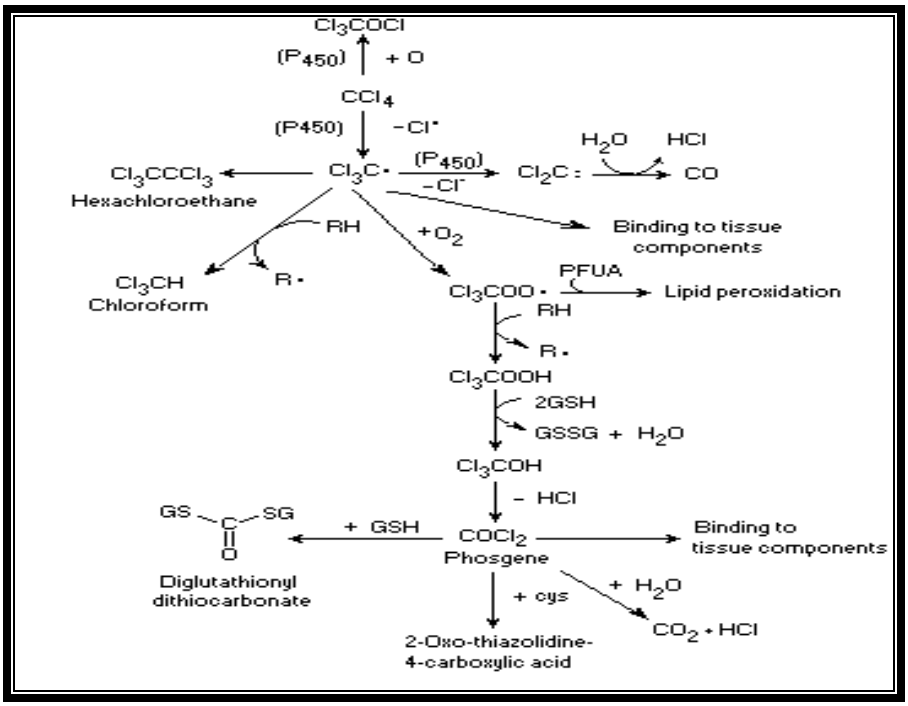

Figura 4: Representação esquemática dos mecanismos de dano oxidativo induzido por tetracloreto de carbono. Adaptado a partir de McGregor e Lang., 1996.

2.4. Organocalcogênios

A partir da década de 30, os organocalcogênios têm sido alvos de interesse para os químicos orgânicos em virtude da descoberta de aplicações sintéticas (Petragnani e col., 1976; Comasseto, 1983) e de propriedades biológicas desses compostos (Parnham & Graf, 1991; Kanda e col., 1999; Nogueira e col., 2004), os quais são importantes intermediários e reagentes muito utilizados em síntese orgânica (Paulmier, 1986; Braga e col., 1996; 1997).

Conseqüentemente, existe o interesse crescente em relação ao desenvolvimento de compostos organocalcogênios que possuam atividade biológica, aplicações farmacológicas com o mínimo de toxicidade e efeitos adversos (Muguesh e col., 2001; Yoshizumi e col., 2002, 2004; Nogueira e col., 2004; Xu e col, 2006). Dentre os compostos orgânicos de selênio estudados no nosso grupo de pesquisa destacam-se o ebselen e o disseleneto de difenila.

2.4.1. Selênio

O elemento selênio foi descoberto em 1817 pelo químico sueco Jöns Jacob Berzelius. Esse elemento químico é um calcogênio do grupo 16 da tabela periódica, podendo apresentar-se sob quatro estados de oxidação: selenato (Se^{+6}), selenito (Se^{+4}), selênio elementar (Se^{0}) e seleneto (Se^{-2}). O Se compartilha propriedades físicas e químicas com o elemento enxofre (S). Esta similaridade permite que o Se substitua o S, promovendo interações Se-S nos sistemas biológicos. Entretanto, as diferenças nas propriedades físico-químicas entre Se e S constituem a base de seus papéis biológicos específicos (Stadtman, 1980).

O selênio é um elemento traço essencial, cuja essencialidade nutricional foi demonstrada em 1957, em ratos (Schwartz e Foltz, 1957). Anteriormente, o único interesse biológico prático para o selênio era que altos níveis deste elemento causavam

toxicidade (Levander e Burk, 1994). 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 tem sido aceita pela comunidade científica, sendo que a Junta de Alimentação e Nutrição da Academia de Ciências dos Estados Unidos propõe uma ingestão diária de 50- 200 μg (Food and Nutrition Board, 1989). Este elemento pode ser encontrado nos seguintes alimentos: castanha-do-pará, alho, cebola, brócolis, cogumelos, cereais, pescados, ovos e carnes (Reilly, 1996; Dumont e col., 2006).

Este calcogênio apresenta um grande número de funções biológicas, sendo a propriedade antioxidante a mais importante (Nogueira e col., 2004). As pesquisas recentes têm procurado estabelecer a função e a biologia molecular de selenoproteínas. Já é conhecido que o selênio está presente como resíduo de selenocisteína no sítio ativo das enzimas glutationa peroxidase (Wingler e Brigelius-Flohé, 1999), tioredoxina redutase (Holmgren, 1985), 5'-deiodinase (Behne e Kyriakopoulos, 1990) e selenoproteína P (Ursini e col., 1990), sendo que a atividade redox do Se tem importância fundamental para o sítio catalítico enzimático.

2.4.2. Biodisponibilidade do selênio

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

Após a absorção, os maiores níveis de selênio estão localizados nos eritrócitos, fígado, baço, coração, unha e esmalte de dentes (Martin e Gerlack, 1972). Na intoxicação crônica em animais, o selênio é depositado principalmente nos rins e fígado, seguido pelo pâncreas, baço e pulmões (Wilber, 1980). A primeira evidência de metabolização de compostos de selênio em animais foi determinada após um longo período de tratamento com selenito de sódio. Os

animais apresentavam odor gárlico característico, que posteriormente demonstrou ter sido causado pelo seleneto de dimetila (Klayman e Gunther, 1973). Esse composto pode ser resultado da detoxificação metabólica de muitos compostos de selênio, a qual envolve uma série de metilações dependentes da S-adenosilmetionina (Hoffman e McConnell, 1986).

O selênio pode ser excretado por três vias: urina, fezes e ar expelido. A excreção urinária deste composto pode auxiliar em casos de intoxicações ou de exposição a altos níveis deste elemento (Valentine e col., 1978). Recentemente, foi demonstrado que dentro dos níveis normais de selênio, ou seja, não tóxicos, a principal forma encontrada na urina é como seleno-açúcar, entretanto, nos caso de doses tóxicas de selênio, o marcador biológico encontrado na urina é o trimetilselenônio (Suzuki e col., 2006). Em indivíduos expostos acidentalmente a altos níveis de Se, pode ser realizada a detecção do composto volátil seleneto de dimetila (Mozier e col, 1988).

2.4.3. Estudo dos compostos orgânicos de selênio

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

Engman e colaboradores (1992) demonstraram que disselenetos e diteluretos de diarila apresentam maior atividade do tipo tiol peroxidase, quando comparados ao ebselen, motivando o uso terapêutico destes compostos.

O ebselen (2-fenil-1,2-benzilsoselenazol-3(2H)-ona) (Figura 5) é um composto orgânico de selênio bastante estudado. Este composto exibe atividade catalítica e propriedades antioxidantes similares à glutationa peroxidase (Parnhan, 1990), possui baixa toxicidade (Parnhan e Graf, 1987), reage com grupos tióis, como a glutationa (Ullrich e col., 1996), inibe a peroxidação lipídica (Parnhan e Graf, 1987; Sies e Artel, 2000; Rossato e col., 2002; Davis e col., 2004; Nowak e col., 2006), inibe a lipoxigenase (Parnhan e Graf, 1987), bloqueia a produção de ânion superóxido e desempenha um papel protetor contra o peroxinitrito (Masumoto e Sies, 1996). Além disso, o ebselen tem sido usado como antioxidante, como neuroprotetor em culturas de neurônios (Osaki e

col., 1997; Tan e col., 1997; Takasago e col., 1997; Kondoh e col., 1999; Imai e col., 2001; Porciúncula e col., 2003), no tratamento clínico de pacientes com isquemia aguda (Yamaguchi e col., 1998; Kondoh e col., 1999), em modelos de Parkinson (Moussaoui e col., 2000) e como antiinflamatório (Parnhan e Graf, 1987; 1991; Walther e col., 1999; Haddad e col., 2002; Mugesh e col., 2001).

O disseleneto de difenila (Figura 6), outro composto orgânico de selênio, demonstrou ser mais ativo como mimético da glutationa peroxidase que o ebselen (Meotti e col., 2004). Estudos conduzidos em nosso laboratório demonstraram que a toxicidade do disseleneto de difenila depende tanto da via de administração quanto da espécie de animal avaliada (ratos ou camundongos). De fato, quando o disseleneto de difenila foi administrado em diferentes doses pela via subcutânea (s.c.) em ratos e camundongos, esse composto não causou nenhum sinal de neurotoxicidade e morte dentro de um período de observação de 72 horas, isso pode ser atribuído a uma baixa taxa de absorção e perda de metabolização do composto. A DL_{50} obtida tanto para ratos quanto para camundongos foi > 500 µmol/kg (156 mg/kg). Por outro lado, quando o disseleneto de difenila foi administrado em diferentes doses pela via intraperitoneal (i.p.) em ratos e camundongos, os parâmetros toxicológicos foram alterados. Por exemplo, a administração i.p. desse composto em camundongos alterou os níveis de creatinina e induziu morte e convulsão, o que pode ser devido à rápida absorção e ao metabolismo de primeira passagem no fígado, a DL_{50} obtida foi de 210 µmol/kg (65 mg/kg). Entretanto, em ratos a administração intraperitoneal do disseleneto de difenila não alterou os parâmetros hepáticos (aspartato aminotransferase - AST; e alanina aminotransferase - ALT) e renais (uréia e creatinina) e a DL_{50} obtida foi de 1200 µmol/kg (374 mg/kg). A partir desses dados, pode-se sugerir que o disseleneto de difenila é mais tóxico em camundongos do que em ratos (Meotti e col., 2003).

Neste contexto, é importante mencionar que o disseleneto de difenila é conhecido por ser menos tóxico que o ebselen. Isso é comprovado, quando ratos são tratados com esses dois compostos pela via intraperitoneal, o valor da DL_{50} para o disseleneto de difenila é cerca de três vezes maior que o ebselen (valores da DL_{50} = 1200 e 400 µmol/kg, respectivamente) (Meotti e col., 2003). Além disso, o ebselen apresenta potência letal semelhante em ratos e camundongos quando administrado pela via i.p. (valores da DL_{50}: 400 e 340 µmol/kg, respectivamente). Por outro lado, quando o

ebselen é administrado pela via subcutânea, ele não induz efeitos tóxicos, tanto para camundongos como para ratos, semelhante como acontece para o disseleneto de difenila.

Com relação à biodistribuiçao em tecidos orgânicos, dados do nosso grupo demonstraram que a exposição crônica ao disseleneto de difenila na dose de 250 μmol/kg aumenta três vezes o total de selênio no cérebro, revelando possíveis evidências que este organocalcogênio é capaz de atravessar a barreira cérebro-sangue devido a sua lipossolubilidade (Maciel e col., 2003; Jacques-Silva e col., 2001) sem induzir alterações nas funções cerebrais avaliadas pelo experimento.

Além da atuação no sistema nervoso central, o disseleneto de difenila possui propriedades farmacológicas mais amplas como: efeitos anti-úlcera (Savegnago e col., 2006), antiinflamatório e antinociceptivo (Nogueira e col., 2003c; Ghislene e col., 2003; Zasso e col., 2005), anti-hiperglicemiante (Barbosa e col., 2006), protege contra a discinesia orofacial induzida por reserpina e haloperidol (Burger e col., 2004, 2006) e pode atuar na facilitação da formação de memória em camundongos (Rosa e col., 2006).

O mecanismo proposto para explicar as propriedades dos compostos de selênio envolve a oxirredução de grupos-SH de moléculas biologicamente ativas (Blais e col., 1972; Young e col., 1981). De fato, diversos trabalhos demonstraram que compostos orgânicos de selênio inibem um grande número de enzimas sulfidrílicas, incluindo a 5-lipoxigenase (Björnstedt e col., 1996), δ-aminolevulinato desidratase (Nogueira e col., 2003a), esqualeno monooxigenase (Gupta e Porter, 2001) e Na^+, K^+- ATPase (Borges e col., 2005). Outros estudos desenvolvidos em nosso laboratório demonstraram que o disseleneto de difenila em altas doses pode causar mal-formação óssea na prole de ratas tratadas durante a gestação (Favero e col., 2005) e no período de organogênese (Weis e col., 2007). Este composto também não pode ser administrado em ratas no período lactacional (Favero e col., 2006), mas não interfere na fertilidade de ratos tratados subcronicamente com este organocalcogênio (Favero e col., 2007).

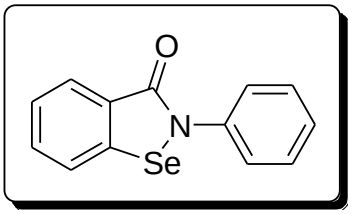

Figura 5- Estrutura química do ebselen

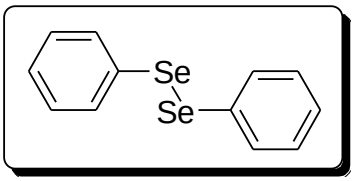

Figura 6- Estrutura química do disseleneto de difenila

2.4.4. Selênio e o Dano Hepático

A associação da importância do selênio na hepatoproteção remonta a meados de 1957, graças a estudos pioneiros desenvolvidos por Schwartz e Foltz, onde foi demonstrado que ratos alimentados com dieta pobre em selênio poderiam desenvolver necrose hepática. Este interessante estudo levou ao reconhecimento que doenças oriundas da privação de nutrientes, poderiam ser causadas por deficiência de selênio na dieta (Oldfield, 1987).

Outra pesquisa relevante demonstrou que a administração oral de ebselen, pode inibir as lipoxigenases em um modelo experimental de indução de hepatite pela administração da endotoxina galactosamina (Wendel e col., 1986). De fato o ebselen demonstrou suas propriedades hepatoprotetoras em diversos modelos de dano hepático, tais como os induzidos por: paracetamol (Li e col., 1994; Rocha e col., 2005), CCl_4 (Wasser e col., 2001), lipopolissacarídeo e *Propionibacterium acnes* (Koyanagi e col., 2001), etanol (Kono e col., 2001), vasoconstrição e etanol (Oshita e col., 1994) e isquemia e reperfusão (Ozaki e col., 1997).

Em células de Kupfer de ratos, o ebselen pode reverter a produção de ânion superóxido e óxido nítrico (Wang e col., 1992), inibindo a cascata de sinalizadores

apoptóticos presentes no dano hepático (fator de necrose tumoral- TNF), interleucina 10 (IL-10) entre outros (Tiegs e col., 1998; Shimohashi e col., 2000).

Com relação ao disseleneto de difenila, estudos conduzidos por Rocha e colaboradores (2005) revelaram que este organocalcogênio pode melhorar a resposta bioquímica hepática em ratos expostos a uma overdose de paracetamol. No ano seguinte, Barbosa e colaboradores (2006) demonstraram que o disseleneto de difenila poderia possuir potencial hepatoprotetor em ratos diabéticos, mas dados na literatura sobre o potencial hepatoprotetor do disseleneto de difenila ainda são muito escassos. Em vista das crescentes descobertas sobre o papel farmacológico de alguns dos organocalcogênios, em especial interesse, o disseleneto de difenila, mais estudos são necessários para elucidar os mecanismos envolvidos no efeito hepatoprotetor deste composto frente a diferentes modelos de dano hepático.

3- OBJETIVOS

Considerando que existem poucos estudos sobre o mecanismo de hepatoproteção induzida pelo disseleneto de difenila pretende-se neste estudo;

- Investigar os efeitos causados pelo disseleneto de difenila frente a diferentes modelos de dano hepático (2-nitropropano, cádmio e tetracloreto de carbono);

- Estudar os possíveis mecanismos envolvidos nos efeitos causados pelo disseleneto de difenila frente a estes modelos experimentais;

4- ARTIGOS CIENTÍFICOS E MANUSCRITOS

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

4.1 – Efeito protetor do disseleneto de difenila no dano hepático agudo induzido por 2-Nitropropano em ratos.

4.1.1 - **Artigo 1**

PROTECTIVE EFFECT OF DIPHENYL DISELENIDE ON ACUTE LIVER DAMAGE INDUCED BY 2-NITROPROPANE IN RATS

Borges, L.P., Borges, V.C., Moro, A.V., Nogueira, C.W., Rocha, J.B.T., Zeni, G*

Toxicology

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Protective effect of diphenyl diselenide on acute liver damage induced by 2-nitropropane in rats

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Received 13 November 2004; received in revised form 30 December 2004; accepted 5 January 2005
Available online 2 February 2005

Abstract

The effect of diphenyl diselenide, $(PhSe)_2$, administration on 2-nitropropane (2-NP)-induced hepatic damage was examined in male rats. Rats were pre-treated with a single dose of diphenyl diselenide (10, 50 or 100 μmol/kg). Afterward, they received only one dose of 2-NP (100 mg/kg body weight dissolved in olive oil). The parameters that indicate tissue damage such as plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transferase (GGT), alpha-fetoprotein (AFP), creatinine and urea were determined. Since toxicity induced by 2-NP is related to oxidative stress, lipid peroxidation was also evaluated. Diphenyl diselenide (100 μmol/kg) significantly reduced plasma ALT, γ-GGT, AFP levels when compared to 2-NP group. Treatment with diphenyl diselenide, at all doses, effectively protects the increase of lipid peroxidation when compared to 2-NP group. Histological examination revealed that 2-NP treatment causes a moderate swelling and degenerative alterations on hepatocytes and diphenyl diselenide (100 μmol/kg) protects against these alterations. Diphenyl diselenide (50 and 100 μmol/kg) significantly decreased the urea level. This study evidences the protective effect of diphenyl diselenide by 2-NP-induced acute hepatic damage.

Keywords: 2-Nitropropane; Organoselenium; Hepatic damage; Liver

1. Introduction

2-Nitropropane (2-NP) has been widely used as a chemical intermediate, a solvent, and a component of inks, paints, varnishes and other coatings (IARC, 1982). This compound is known to be an acute hepatotoxicant (Zitting et al., 1981) and a potent hepatocarcinogen in rodents when administered either by inhalation (Lewis et al., 1979) or orally (Fiala et al., 1987). 2-NP has been also reported to be carcinogenic to humans (Petrelli et al., 1993) and animals (Lewis et al., 1979; Fiala et al., 1989). The mechanism by

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doi:10.1016/j.tox.2005.01.002

which 2-NP causes toxicity is not completely elucidated, but accumulating evidence suggests that generation of reactive oxygen species via the metabolism of 2-NP-nitronate to acetone and nitrite plays an important role for the carcinogenic effect of 2-NP (Roscher et al., 1990; Halliwell and Gutteridge, 1990). It has been suggested that 2-NP metabolism may also generate nitric oxide (NO) radicals (Kohl et al., 1995).

The rapid growth, in recent years, of the role of reactive oxygen species in pathology has brought with new ideas for the therapy of a variety of diseases. In this way, several reports have appeared describing the antioxidant activity of ebselen (Rossato et al., 2002) and other organoselenium compounds (Nogueira et al., 2004; Meotti et al., 2004) in different experimental models. In fact, selenium is known to be an essential biological trace element that plays a crucial role as an integral component of several enzymes with antioxidant properties, including gluthathione peroxidase (Flohé et al., 1973; Rotruck et al., 1973; Wilson et al., 1989) and several other selenoproteins (Linder, 1990; Ursini et al., 1982). Studies suggested that low selenium status may increase the risk of oxidative damage and cancer (Rayman, 2000). As well, selenium supplementation induces chemo-protective and anti-cancer activities (Medina et al., 1983). Diphenyl diselenide, an organoselenium compound, has been recently reported as a hepatoprotector compound in diabetic rats (Nogueira et al., 2004). Other authors have also described the potential pharmacological profile of organoselenium compounds (Andersson et al., 1994; Nogueira et al., 2003; Meotti et al., 2004; Nogueira et al., 2004).

Based on these facts, the present study investigated the effect of diphenyl diselenide at different doses on 2-NP-induced acute hepatic damage in rats.

2. Material and methods

2.1. Chemicals

Diphenyl diselenide (Fig. 1) was synthesized according to literature methods (Paulmier, 1986) and was dissolved in olive oil. Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed that diphenyl diselenide presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical

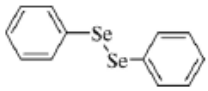

Fig. 1. Chemical structure of diphenyl diselenide.

purity of diphenyl diselenide (99.9%) was determined by GC/HPLC. 2-Nitropropane was obtained from Sigma. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.2. Animals

Male adult albino Wistar rats (200–250 g) from our own breeding colony were used. The animals were kept in separate animal rooms, on a 12 h light:12 h dark cycle, at a room temperature of 22 °C and with free access to food and water. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, School of Medicine, Veterinary, and Animal Science of the University of Sao Paulo, Brazil.

2.3. Exposure

A group of six animals was usually tested in each experiment. The control group (group 1) received only vehicle (olive oil, 5 ml/kg). Treated rats were injected intraperitoneally with a single dose of diphenyl diselenide (10, 50 or 100 μmol/kg) (groups 3–5) and 24 h later they were injected intraperitoneally with 2-NP (100 mg/kg body weight dissolved in olive oil) (groups 2, 6–8). The 2-NP dose and current protocol were chosen according to previous reports (Fiala et al., 1989; Guo et al., 1990; Robbiano et al., 1991), which have found liver damage after a single dose of 2-NP.

Twenty four hours after 2-NP injection, rats were slightly anesthetized with ether for blood collect. Blood was collected by heart puncture in tubes containing heparin. Plasma was obtained by centrifugation at 2000 × *g* for 10 min (hemolyzed plasma was discarded). All groups were killed by decapitation and the liver, kidney and spleen were dissected.

The protocol of rat treatments is given below:

Group 1 Olive oil (i.p.) plus olive oil (5 ml/kg, i.p.).

Group 2 Olive oil (5 ml/kg, i.p.) plus 2-NP (100 mg/kg, i.p.).
Group 3 Diphenyl diselenide (10 μmol/kg, i.p.) plus olive oil (5 ml/kg, i.p.).
Group 4 Diphenyl diselenide (50 μmol/kg, i.p.) plus olive oil (5 ml/kg, i.p.).
Group 5 Diphenyl diselenide (100 μmol/kg, i.p.) plus olive oil (5 ml/kg, i.p.).
Group 6 Diphenyl diselenide (10 μmol/kg, i.p.) plus 2-NP (100 mg/kg, i.p.).
Group 7 Diphenyl diselenide (50 μmol/kg, i.p.) plus 2-NP (100 mg/kg, i.p.).
Group 8 Diphenyl diselenide (100 μmol/kg, i.p.) plus 2-NP (100 mg/kg, i.p.).

2.4. Plasma enzymes

Plasma enzymes AST (aspartate aminotransferase), ALT (alanine aminotransferase) and GGT (γ-glutamyl transferase) were used as the biochemical markers for the early acute hepatic damage (Reitman and Frankel (1957), using a commercial Kit (LABTEST, Diagnostica S.A., Minas Gerais, Brazil).

2.5. Plasma creatinine and urea

Renal function was analyzed using a commercial Kit (LABTEST, Diagnostica S.A., Minas Gerais, Brazil) by determining plasma urea (Mackay and Mackay, 1927) and creatinine (Jaffe, 1886).

2.6. Plasma alpha-fetoprotein

Hepatic tumor marker was analyzed by determining plasma alpha-fetoprotein by enzyme-immunoassay method (ABBOTT, USA). Data are expressed as ng/dl.

2.7. Lipid peroxidation

Lipid peroxidation was performed by the formation of TBARS (thiobarbituric acid reactive species) during an acid-heating reaction as previously described by Draper and Hadley (1990). Briefly, the samples were mixed with 1 ml of 10% TCA and 1 ml of 0.67% thiobarbituric acid subsequently they were heated in a boiling water bath for 15 min. TBARS were determined by the absorbance at 535 nm and were expressed as nmol MDA/mg protein.

2.8. Evaluation of hepatic damage incidence

The liver was carefully inspected for the detection of visible macroscopic lesions such as abnormal surface and color and presence of visible nodes. Thus, the hepatic damage incidence (%) was determined by the presence or absence of such visible abnormalities. All macroscopic lesions were examined by histopathology.

2.9. Histological evaluation

At sacrifice, all rats were slightly anesthetized and subjected to a through necropsy evaluation. Organ weight for liver and kidney was recorded, and tissues were saved and fixed in 10% formalin. For light microscopy examination, tissues were embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin.

2.10. Statistical analysis

Data were analyzed by analysis of variance (ANOVA) (2-nitropropane × diphenyl diselenide), followed by Duncan's Multiple Range Test when appropriate. Differences between groups were considered significant when $p < 0.05$. Main effects are presented only when the higher (second) order interaction was non-significant.

3. Results

3.1. Effect of diphenyl diselenide on incidence of hepatic damage

2-NP-treated group presented 92% the incidence of hepatic damage when compared to control group. Pre-treatment with diphenyl diselenide (10, 50 or 100 μmol/kg) protected effectively against hepatic damage caused by 2-NP about 25, 50 and 84%, respectively. Diphenyl diselenide, at all tested doses, did not induce hepatic damage (Table 1).

3.2. Urea and creatinine levels

Two-way ANOVA of urea level yield a significant 2-NP × $(PhSe)_2$ interaction. Post-hoc comparisons demonstrated that 2-NP increased ($p < 0.0024$)

Table 1
Effect of diphenyl diselenide on toxicological parameters after a single intraperitoneal administration of 2-NP in rats

Groups	Hepatic damage incidence (%)	Urea (mg/dl)	ALT (U/l)	GGT (U/l)	TBARS[a]
Control	0	31.4 ± 8.1	43.5 ± 9.1	4.6 ± 2.7	13.4 ± 4.4
2-NP	92	42.6 ± 6.6*	59.0 ± 8.2*	15.6 ± 3.5*	28.1 ± 6.5*
Se 10 (μmol/kg)	0	32.0 ± 2.8	26.0 ± 1.4	2.5 ± 0.7	14.6 ± 1.5
Se 50	0	33.5 ± 0.7	32.0 ± 1.4	2.0 ± 0.0	14.1 ± 1.7
Se 100	0	27.1 ± 2.1	42.8 ± 6.4	8.0 ± 3.0	13.1 ± 0.8
Se 10 + 2-NP	75	40.0 ± 5.6*	63.0 ± 2.4*	13.2 ± 0.9*	17.2 ± 1.8†
Se 50 + 2-NP	50	32.5 ± 3.5†	62.0 ± 5.6*	16.0 ± 2.8*	12.7 ± 2.7†
Se 100 + 2-NP	16	28.4 ± 2.9†	45.2 ± 7.1†	6.6 ± 2.0†	13.1 ± 2.1†

Data are expressed as mean ± S.D. of six animals per group.
[a] Data of hepatic TBARS are presented as nmol MDA/mg protein.
* Denoted $p < 0.05$ as compared to control group (ANOVA/Duncan).
† Denoted $p < 0.05$ as compared to 2-NP group (ANOVA/Duncan).

urea level when compared to control group. Diphenyl diselenide at 10 μmol/kg failed in reducing the increase of urea level induced by 2-NP (Table 1). Pre-treatment with 50 or 100 μmol/kg of diphenyl diselenide significantly decreased the urea level when compared to 2-NP group (Table 1).

Plasma creatinine level was not altered in all tested groups (data not shown).

3.3. ALT, AST and GGT activities

Two-way ANOVA of ALT activity yield a significant 2-NP × $(PhSe)_2$ interaction. Post-hoc comparisons demonstrated that 2-NP increased ($p < 0.0000$) ALT activity. Diphenyl diselenide (10 and 50 μmol/kg) administered in rats did not protect against the increase of ALT (Table 1) activity induced by 2-NP. Treatment with 100 μmol/kg diphenyl diselenide effectively protects the increase of ALT activity (Table 1).

According to the two-way ANOVA of GGT activity there was a significant 2-NP × $(PhSe)_2$ interaction ($p < 0.000005$). In fact, there was a significantly increase ($p < 0.05$, Duncan's multiple range test) in plasma GGT activity in rats treated with 2-NP when compared to control group. Only the high dose of diphenyl diselenide was effective to protect the increase on GGT activity (Table 1).

Plasma AST activity was unchanged on all tested groups (data not shown).

3.4. Plasma alpha-fetoprotein

Two-way ANOVA of AFP level yield a significant 2-NP × $(PhSe)_2$ interaction. Post-hoc comparisons demonstrated that 2-NP significant increased AFP level (8.06 ± 0.37 ng/dl, $p < 0.00005$) when compared to control group (0.63 ± 0.15 ng/dl). Diphenyl diselenide 50 μmol/kg (5.40 ± 0.85 ng/dl) or 100 μmol/kg (1.70 ± 0.91 ng/dl) plus 2-NP significantly reduced AFP level when compared to 2-NP group. AFP level on diphenyl diselenide 10 μmol/kg plus 2-NP group (7.40 ± 0.62 ng/dl) did not differ from 2-NP group. Given alone, diphenyl diselenide at all tested doses did not alter AFP level (data not shown).

3.5. Lipid peroxidation

Two-way ANOVA of TBARS levels yield a significant 2-NP × $(PhSe)_2$ interaction. Post-hoc comparisons demonstrated that 2-NP increased ($p < 0.0063$) lipid peroxidation in liver (2.0-fold higher than the corresponding control group). Treatment with $(PhSe)_2$ was effective in protecting TBARS status towards to control level (Table 1). Renal TBARS levels were unchanged on all tested groups (data not shown).

3.6. Histological evaluation

Histological examination revealed that 2-NP treatment causes a moderate swelling and degenerative alterations on hepatocytes (Fig. 3) when compared to control group (Fig. 2). Degenerative changes were not evident on group 8 (Figs. 4 and 5).

4. Discussion

The present study produced convincing evidence that diphenyl diselenide has a protective effect against

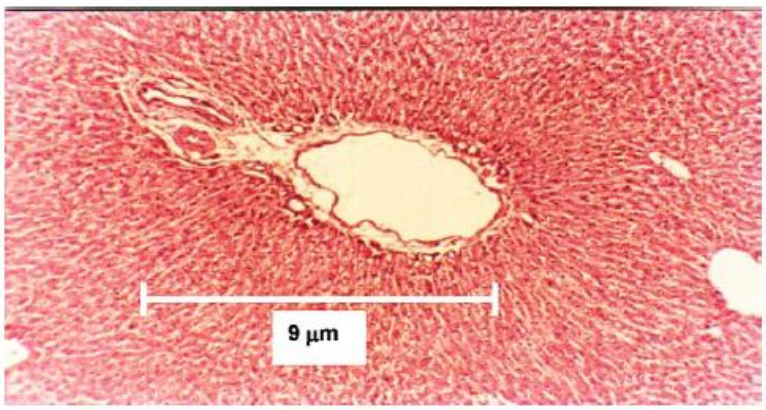

Fig. 2. Histological evaluation of liver from control group (20×).

2-NP-induced acute liver damage. Accordingly, several research groups have demonstrated that liver damage is a therapeutic target of selenorganic compounds, as well as, the various clinical conditions in which hydroperoxides play a role (Nogueira et al., 2004).

Diphenyl diselenide at dose of 100 µmol/kg protected effectively against the incidence of acute liver damage caused by 2-nitropropane. As the main alterations visualized in the animals exposed to 2-NP were protected by diphenyl diselenide (100 µmol/kg), histological blades of liver from these animals were produced. Microscopic histological evaluation confirmed hepatic damage with moderate swelling and degenerative alterations on hepatocytes (Fig. 3), which were similar to the acute effects of 2-NP observed by others (Zitting et al., 1981). Thus, liver damage observed during the external examination (Table 1) was confirmed after histological evaluation (compare Fig. 2 to Fig. 3). Animals treated with diphenyl diselenide did not present histological alterations (compare Figs. 4 and 5 to Fig. 3) confirming a hepato-protective effect of this organoselenium compound. In addition, diphenyl diselenide, at 100 µmol/kg, reduced the increase of ALT, GGT and AFP induced by 2-NP. In fact, 2-NP increases the plasma ALT and GGT activities,

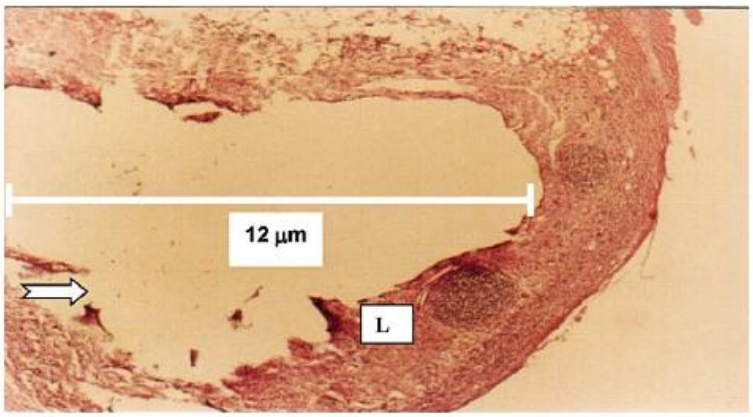

Fig. 3. Histological evaluation of liver from 2-NP (100 mg/kg) group. There is a marked cellular damage (arrow) and the central vein has disappeared. Lymphonodes are present in the posterior lobe (L) (20×).

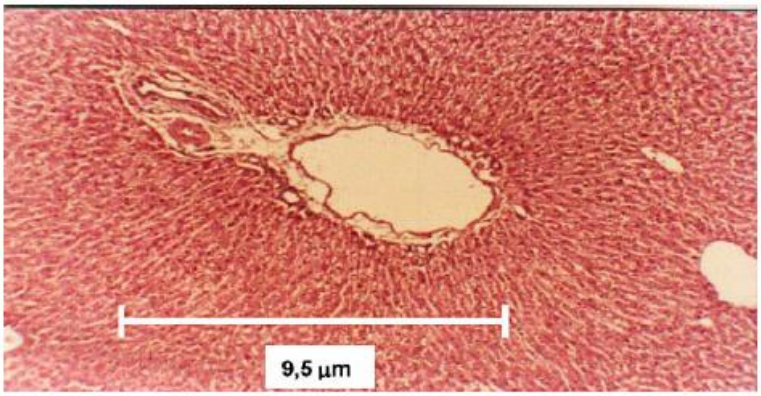

Fig. 4. Histological evaluation of liver from 2-NP (100 mg/kg) plus 100 μmol/kg diphenyl diselenide group. The liver is normal (20×).

which are indicative of hepatotoxicity (Prasada and Hariharaq, 1991). The increase of AFP, characterizing acute liver damage, has been also reported by others (Machle et al., 1940). Consistent with these findings, recently, our group has reported a hepato-protective effect of diphenyl diselenide in diabetic rats (Nogueira et al., 2004). Of note, the liver has been implicated as the principal site of 2-NP metabolism (Dequit et al., 1972), which generates acetone and nitrite by the microssomal cytochrome P-450 system (Ulrich et al., 1978). The metabolism of 2-NP could also produce NO radicals (Kohl et al., 1995), which probably induces the hepatic damage.

Since diphenyl diselenide (10 and 50 μmol/kg) reduced 25 and 50%, respectively, the incidence of hepatic damage and did not protect against the increase of ALT and GGT caused by 2-NP, this study also suggested that hepato-protective effect of diphenyl diselenide was dose-dependent.

In addition, diphenyl diselenide (50 and 100 μ mol/kg) protected against acute renal damage evidenced by a decrease on urea levels on animals treated

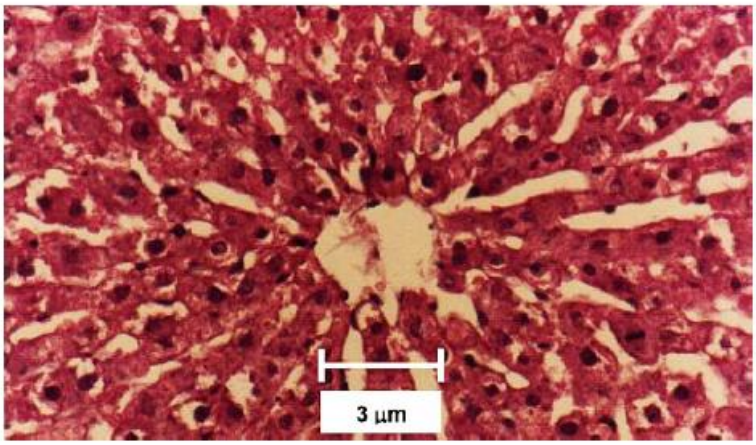

Fig. 5. Histological evaluation of liver from 2-NP (100 mg/kg) plus 100 μmol/kg diphenyl diselenide. There is no alteration on hepatocytes (100×).

with this organoselenium compound. These findings are in accordance to Kim and collaborators (1998) who demonstrated that 2-NP is a potent nephrotoxin which induces kidney damage.

Concerning the protective mechanisms of diphenyl diselenide, our data clearly demonstrated that antioxidative properties of diphenyl diselenide are primarily involved on 2-NP-induced liver damage. In fact, diphenyl diselenide, at all tested doses, was able to protect the increase on TBARS levels induced by 2-NP administration. Since, the biochemical and hematological changes occur first than the alterations on the cellular structure, diphenyl diselenide, even at low doses, could be protecting the early damage induced by 2-NP.

Accordingly, the changes related to the oxidative stress, such as oxidative liver damage, were known to be eliminated by selenium administration; possibly due, in part, to scavenging the intermediates derived from 2-NP metabolism, including nitrogen oxide or their radical derivatives (Kohl et al., 1995). Otherwise, data from our group have demonstrated that diphenyl diselenide, at doses similar or upper to those used in this study, was safety and did not alter the renal and hepatic functions (Meotti et al., 2003). As well, diphenyl diselenide demonstrated a hepato-protective effect (Nogueira et al., 2004), antioxidant (Meotti et al., 2004), anti-inflammatory (Nogueira et al., 2003) and antiulcer properties (Nogueira et al., 2004).

In conclusion, this study demonstrated that the pre-treatment with diphenyl diselenide prevented hepatotoxicity and cellular damage in the rat liver after a single dose of 2-NP and suggested that the administration of 100 μmol/kg may be effective in mitigating the hazards from 2-NP exposure. These data may provide practical indications about the benefits of diphenyl diselenide administration to protect human health from hazards by a variety of environmental toxicants, which induce hepatic damage.

Acknowledgements

The financial support by Fundacao de Amparo a Pesquisa do Rio Grande do Sul (FAPERGS), Coordenacao de Aperfeicoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq) is gratefully acknowledged. J.B.T.R. and G.Z. are the recipient of CNPq fellowships.

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4.2 – A administração oral de disseleneto de difenila protege contra o dano hepático induzido por cádmio em ratos.

4.2.1 – **Artigo 2**

ORAL ADMINISTRATION OF DIPHENYL DISELENIDE PROTECTS AGAINST CADMIUM-INDUCED LIVER DAMAGE IN RATS

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Chemico-Biological Interactions

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Oral administration of diphenyl diselenide protects against cadmium-induced liver damage in rats

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Received 12 June 2007; received in revised form 6 September 2007; accepted 7 September 2007

Abstract

Cadmium is an environmental toxic metal implicated in human diseases. In the present study, the effect of diphenyl diselenide, $(PhSe)_2$, on sub-chronic exposure with cadmium chloride ($CdCl_2$) was investigated in rats. Male adult Swiss albino rats received $CdCl_2$ (10 μmol/kg, orally) and $(PhSe)_2$ (5 μmol/kg, orally) for a period of 30 days. A number of parameters were examined as indicators of toxicity, including hepatic and renal damage, glucose and glycogen levels and markers of oxidative stress. Cadmium content, liver histology, δ-aminolevulinate dehydratase (δ-ALA-D) activity, metallothionein (MT) levels were also evaluated. Cadmium content determined in the tissue of rats exposed to $CdCl_2$ provides evidence that the liver is the major cadmium target where $(PhSe)_2$ acts. The concentration of cadmium in liver was about three fold higher than that in kidney, and $(PhSe)_2$ reduced about six fold the levels of this metal in liver of rats exposed. Rats exposed to $CdCl_2$ showed histological alterations abolished by $(PhSe)_2$ administration. $(PhSe)_2$ administration ameliorated plasma malondialdehyde (MDA) levels, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and gamma-glutamyl transferase (GGT) activities increased by $CdCl_2$ exposure. Urea and bilirubin levels increased by $CdCl_2$ exposure were also reduced by $(PhSe)_2$. In conclusion, this study demonstrated that co-treatment with $(PhSe)_2$ ameliorated hepatotoxicity and cellular damage in rat liver after sub-chronic exposure with $CdCl_2$. The proposed mechanisms by which $(PhSe)_2$ acts in this experimental protocol are its antioxidant properties and its capacity to form a complex with cadmium.

Keywords: Cadmium; Selenium; Diphenyl diselenide; Liver damage; Oxidative stress

1. Introduction

Cadmium is one of the most important toxic chemicals due to its increasing level in the environment as a result of tobacco smoking, industrial and agricultural practices [1,2]. It has a very long biological half-life (10–30 years) in humans and its toxicity is dependent on the route, dose and duration of exposure [2–4]. Acute cadmium intoxication induced primarily hepatic and testicular damage whereas, chronic exposure resulted in renal injury and osteotoxicity [4–6]. Parenteral administration of cadmium in rats caused a severe hepatic injury in the form of hepatocellular necrosis [7].

The molecular mechanism that may be responsible for the toxicity of cadmium involves oxidative stress by disturbing the antioxidant defense systems and by producing reactive oxygen species [8–10]. In view of the fact

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doi:10.1016/j.cbi.2007.09.005

that cadmium is a non-redox metal, it always adopts a single oxidation state and is not a strong inducer of reactive oxygen species. The major mechanism behind the case is the disruption of electron transfer chain and the induction of mitochondrial reactive oxygen species (ROS) [10]. Therefore, some authors have postulated that antioxidants should be one of the important components of an effective treatment of cadmium poisoning [5,11,12].

The discovery of the pharmacological potential of organoselenium compounds [13–18], especially diphenyl diselenide, $(PhSe)_2$, motivates the interest in its mechanism of action, considering the antioxidant property and the formation of a complex with metals in the main idea of the discussion. The antioxidant activity explains some protective effects of $(PhSe)_2$ on oxidative models of damage in different tissues [19–24]. Previous studies have reported the formation of a complex between cadmium and $(PhSe)_2$ [24]. This study reports the effect of $(PhSe)_2$ on sub-chronic exposure with $CdCl_2$ in rats.

2. Materials and methods

2.1. Chemicals

Cadmium chloride ($CdCl_2$) was obtained from Merck (Darmstadt, Germany). 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 [25]. 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 $(PhSe)_2$ (99.9%) was determined by GC/HPLC. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.2. Animals

Male adult Wistar rats (200–250 g) from our own breeding colony were used. The animals were kept in a separate animal room, on a 12 h light/dark cycle, at a room temperature of 22 ± 2 °C and with free access to food and water. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, the Federal University of Santa Maria, Brazil.

2.3. Experimental procedure

Rats were orally administered by gavage every other day with $CdCl_2$ and $(PhSe)_2$ for 30 days. A group of six rats was usually tested in each experimental group. The rats received $CdCl_2$ (10 μmol/kg, dissolved in saline, 1 mL/kg) [12] and 30 min later $(PhSe)_2$ (5 μmol/kg, dissolved in canola oil, 1 mL/kg). The dose of $(PhSe)_2$ was based on previous studies of our research group.

The protocol of rat treatment is given below:

- Group 1: saline + canola oil;
- Group 2: $CdCl_2$ 10 μmol/kg + canola oil;
- Group 3: saline + $(PhSe)_2$ 5 μmol/kg;
- Group 4: $CdCl_2$ 10 μmol/kg + $(PhSe)_2$ 5 μmol/kg.

At 24 h after the last $CdCl_2$ injection, the blood samples were collected directly from the ventricle of the heart in anesthetized animals. Subsequently, rats were euthanized by decapitation, liver was removed and rapidly homogenized in 50 mM Tris–HCl, pH 7.5 (1/10, w/v) and centrifuged at 2400 × *g* for 15 min.

2.4. Hepatic and cellular markers of damage

Plasma enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT) and lactate dehydrogenase (LDH) were assayed spectophotometrically according to Reitman and Frankel [26], IFCC [27] and Bergmeyer [28], using a commercial kit (LABTEST, Diagnostica S.A., Minas Gerais, Brazil).

2.4.1. Bilirubin contents

Estimation of plasma total, direct and indirect bilirubin contents were assayed spectrophotometrically according to Malloy and Evelyn [29], using a commercial kit (LABTEST, Diagnostica S.A., Minas Gerais, Brazil).

2.5. Renal markers of damage

Renal function was analysed using a commercial kit (LABTEST, Diagnostica S.A., Minas Gerais, Brazil) by determining urea [30] and creatinine levels in plasma [31].

2.6. Glucose levels

Plasma glucose was assayed spectrophotometrically according to Blaedel and Uhl [32], using a commercial kit (LABTEST, Diagnostica S.A., Minas Gerais, Brazil).

2.7. Glycogen assay

Hepatic glycogen content was assayed by the method described by Krisman [33]. Briefly, a known amount of

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liver was digested in 2 mL of 30% KOH solution. Followed 10 min in boiling water bath, 2 mL of ethanol was added to the tubes to precipitate glycogen. After precipitation, glycogen was resuspended in 0.2 mL 5N HCl and 0.8 mL distilled water. The glycogen content was measured with iodine reagent at 460 nm and expressed as gram of glycogen/100 g of liver.

2.8. Malondialdehyde (MDA) levels

An aliquot (200 μL) of plasma individual samples was used to carry out MDA assay. This procedure was used for samples from all groups. The method used for analysis was automated ELISA-IMMUNO-ASSAY.

2.9. δ-Aminolevulinate dehydratase (δ-ALA-D) activity

Hepatic δ-ALA-D activity was assayed by the method of Sassa [34] by measuring the rate of product (porphobilinogen) formation except that 1 M potassium phosphate buffer, pH 6.8 and 12 mM of aminolevulinic acid (ALA) were used. Incubations were carried out for 30 min at 39 °C. The reaction product was determined using modified Ehrlich's reagent at 555 nm, with a molar absorption coefficient of $6.1 \times 10^4\ M^{-1}$ for the Ehrlich–porphobilinogen salt.

2.10. Catalase activity

Hepatic catalase activity was determined by the decomposition of H_2O_2 according to Aebi [35].

2.11. Superoxide dismutase activity

Superoxide dismutase (SOD) activity in liver homogenate was assayed spectrophotometrically as described by Misra and Fridovich [36]. This method is based on the capacity of SOD in inhibiting 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 epinephrine autoxidation by 50% at 26 °C.

2.12. Glutathione S-transferase activity

Hepatic glutathione *S*-transferase (GST) activity was assayed through the conjugation of glutathione with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm as described by Habig et al. [37].

2.13. Ascorbic acid levels

Hepatic ascorbic acid determination was performed as described by Jacques-Silva et al. [38]. Protein (liver) was precipitated in 10 volumes of a cold 4% trichloroacetic acid solution. An aliquot of homogenized sample (300 mL), in a final volume of 1 mL of the solution, was incubated at 38 °C for 3 h, then 1 mL H_2SO_4 65% (v/v) was added to the medium. The reaction product was determined using color reagent containing 4.5 mg/mL dinitrophenyl hydrazine and $CuSO_4$ (0.075 mg/mL).

2.14. Nonprotein thiols (NPSH) content

Hepatic NPSH levels were determined by the method of Ellman [39]. A sample of supernatant (500 μL) was mixed (1:1) with 10% trichloroacetic acid (500 μL). After centrifugation, the protein pellet was discarded and free –SH groups were determined in a clear supernatant. An aliquot (100 μL) of supernatant was added in a 1 M potassium phosphate buffer (850 μL), pH 7.4, and 10 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) (50 μL). The color reaction was measured at 412 nm.

2.15. Metallothionein (MT) content

Metallothionein content determination of liver was assayed according to the method of Viarengo et al. [40] as modified by Petrovic et al. [41]. Aliquots of 1 mL of supernatant were added with 1.05 mL of cold (−20 °C) absolute ethanol and 80 μL of chloroform; the samples were then centrifuged at $6000 \times g$ for 10 min. The collected supernatant was combined with three volumes of cold ethanol (−20 °C), maintained at −20 °C for 1 h and centrifuged at $6000 \times g$ for 10 min. The metallothionein-containing pellets were then rinsed with 87% ethanol and 1% chloroform and centrifuged at $6000 \times g$ for 10 min. The metallothionein content in the pellet was evaluated using the colorimetric method with Ellman's reagent. The pellet was resuspended in 150 μL 0.25 M NaCl and subsequently 150 μL 1N HCl-containing EDTA 4 mM were added to the sample. A volume of 4.2 mL 2 M NaCl-containing 0.43 mM DTNB buffered with 0.2 M Na–phosphate, pH 8.0 [39] was then added to the sample at room temperature. The sample was finally centrifuged at $3000 \times g$ for 5 min and the supernatant absorbance was evaluated at 412 nm.

2.16. Protein determination

Protein was measured by the method of Lowry et al. [42] using bovine serum albumin as standard.

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2.17. Cadmium content

Cadmium concentrations in plasma, kidney and liver were analyzed by atomic absorption spectrometry. The samples (kidney and liver) were prepared with nitric acid (65%) for total dissolution. A pool of samples, containing an aliquot (200 μL) of individual samples, was used to evaluate cadmium content. Three measurements on a pool of samples for each experimental group were performed.

2.18. Histopathology

At sacrifice, all rats were slight anesthetized and subjected to a thorough necropsy evaluation. Organ weight for liver was recorded, and tissues were saved and fixed in 10% formalin. For light microscopy examination, tissues were embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin. All groups treated were examined by histopathology ($n = 3$ per each group).

2.19. Statistical analysis

Data are expressed as mean ± S.D. Statistical analysis was performed to compare treated groups to respective control groups using a two-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. Hepatic and cellular markers of damage

These parameters were carried out with the aim to evaluate if $CdCl_2$ and $(PhSe)_2$ exposure alters markers of liver and cellular damage.

3.1.1. AST and ALT activities

A significant $CdCl_2 \times (PhSe)_2$ interaction for AST activity was observed ($F_{1,12} = 44.38$, $p < 0.001$) (Fig. 1A). Post hoc comparisons demonstrated that rats exposed to $CdCl_2$ presented an increase (31%) in AST activity. $(PhSe)_2$ administration was effective in ameliorating the AST activity increased by $CdCl_2$.

Two-way ANOVA of ALT activity yielded a significant $CdCl_2 \times (PhSe)_2$ interaction ($F_{1,15} = 29.14$, $p < 0.001$) (Fig. 1B). Post hoc comparisons demonstrated that rats exposed to $CdCl_2$ presented an increase (69%) in ALT activity. $(PhSe)_2$ administration abolished the effect of $CdCl_2$. ALT activity was reduced (16%) by administration of $(PhSe)_2$ alone.

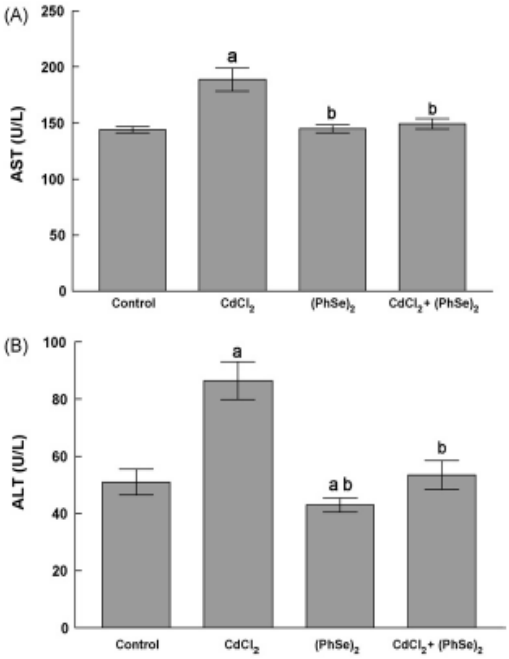

Fig. 1. Effect of $(PhSe)_2$ on plasma AST (A) and ALT (B) activities of rats exposed to $CdCl_2$. Data are reported as mean ± S.D. of six animals per group. (a) $p < 0.05$ as compared to the control group (two-way ANOVA/Duncan) and (b) $p < 0.05$ as compared to the $CdCl_2$ group (two-way ANOVA/Duncan).

3.1.2. LDH, ALP and GGT activities

Two-way ANOVA of plasma LDH activity revealed a significant main effect of $CdCl_2$ ($p < 0.05$) and $(PhSe)_2$ ($p < 0.05$) (Fig. 2A). Post hoc comparisons demonstrated that rats exposed to $CdCl_2$ presented an increase (47%) in LDH activity. The increase in LDH activity induced by $CdCl_2$ was abolished by $(PhSe)_2$ administration. Rats that received $(PhSe)_2$ presented a reduction (39%) in LDH activity.

A significant main effect of $CdCl_2$ ($p < 0.05$) and $(PhSe)_2$ ($p < 0.05$) in plasma ALP activity was observed (Fig. 2B). Results demonstrated that $CdCl_2$ exposure caused an increase (45%) in ALP activity. $(PhSe)_2$ administration ameliorating the ALP activity increased by $CdCl_2$. ALP activity was reduced (37%) by administration of $(PhSe)_2$.

Two-way ANOVA of plasma GGT activity yielded a significant $CdCl_2 \times (PhSe)_2$ interaction ($F_{1,15} = 34.83$, $p < 0.001$) (Fig. 2C). Post hoc comparisons demonstrated that $CdCl_2$ exposure significantly increased (2.5 times higher when compared to the control group) GGT activity. The increase in GGT activity induced by $CdCl_2$ was abolished by $(PhSe)_2$ administration (Fig. 2C).

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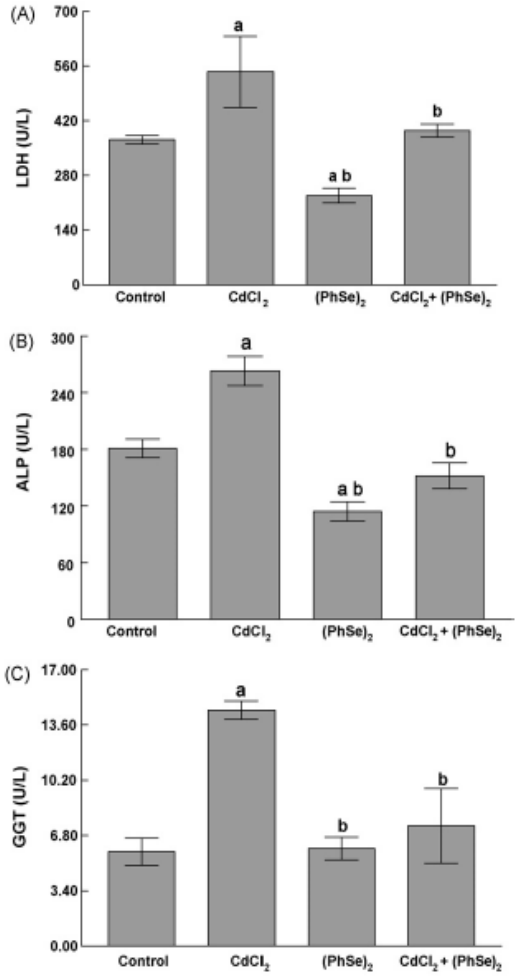

Fig. 2. Effects of $(PhSe)_2$ on plasma LDH (A), ALP (B) and GGT (C) activities of rats exposed to $CdCl_2$. Data are reported as mean ± S.D. of six animals per group. (a) $p < 0.05$ as compared to the control group (two-way ANOVA/Duncan) and (b) $p < 0.05$ as compared to the $CdCl_2$ group (two-way ANOVA/Duncan).

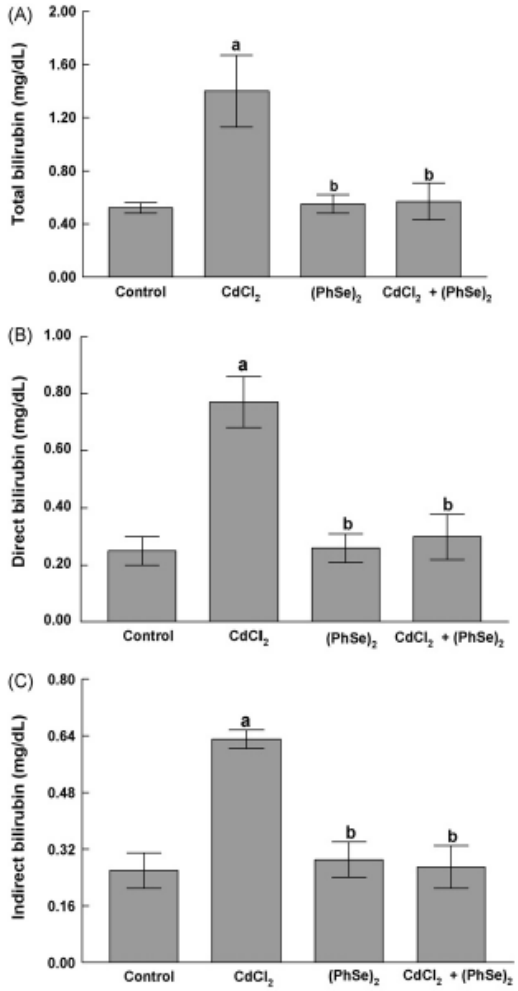

Fig. 3. Effects of $(PhSe)_2$ on total (A), direct (B) and indirect (C) plasma bilirubin content of rats exposed to $CdCl_2$. Data are reported as mean ± S.D. of six animals per group. (a) $p < 0.05$ as compared to the control group (two-way ANOVA/Duncan) and (b) $p < 0.05$ as compared to the $CdCl_2$ group (two-way ANOVA/Duncan).

3.2. Bilirubin content

Two-way ANOVA of plasma total bilirubin levels yielded a significant $CdCl_2 \times (PhSe)_2$ interaction ($F_{1,15} = 39.55$, $p < 0.001$) (Fig. 3A). Post hoc comparisons demonstrated that rats exposed to $CdCl_2$ presented an increase (2.7 times higher when compared to the control group) in total bilirubin levels (Fig. 3A). $(PhSe)_2$ administration was effective in abolished the increase of total bilirubin content induced by $CdCl_2$ (Fig. 3A).

A significant $CdCl_2 \times (PhSe)_2$ interaction in plasma direct bilirubin levels was observed ($F_{1,15} = 53.15$, $p < 0.001$) (Fig. 3B). $CdCl_2$ exposure caused a significantly increase (3.0 times higher than in the control group) in direct bilirubin levels. $(PhSe)_2$ administration ameliorating the direct bilirubin content increased by $CdCl_2$ (Fig. 3B).

Two-way ANOVA of plasma indirect bilirubin levels yielded a significant $CdCl_2 \times (PhSe)_2$ interaction ($F_{1,14} = 52.02$, $p < 0.001$) (Fig. 3C). Post hoc comparisons showed that $CdCl_2$ exposure increased (2.4 times higher than in the control group) indirect bilirubin levels. The increase in indirect bilirubin content induced

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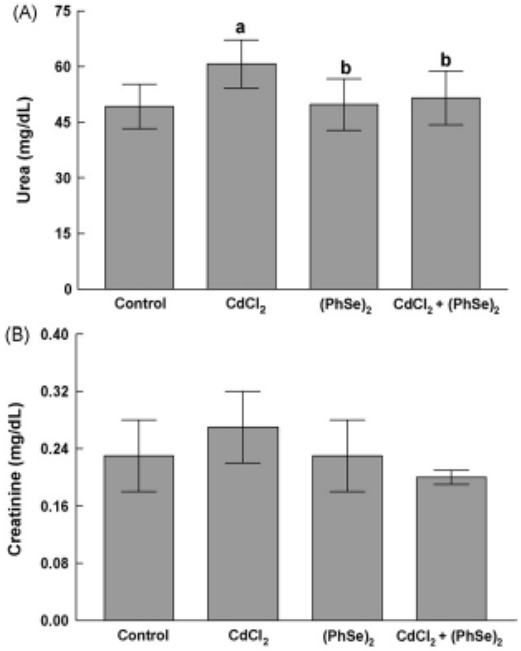

Fig. 4. Effect of $(PhSe)_2$ on plasma urea (A) and creatinine (B) levels of rats exposed to $CdCl_2$. Data are reported as mean ± S.D. of six animals per group. (a) $p < 0.05$ as compared to the control group (two-way ANOVA/Duncan) and (b) $p < 0.05$ as compared to the $CdCl_2$ group (two-way ANOVA/Duncan).

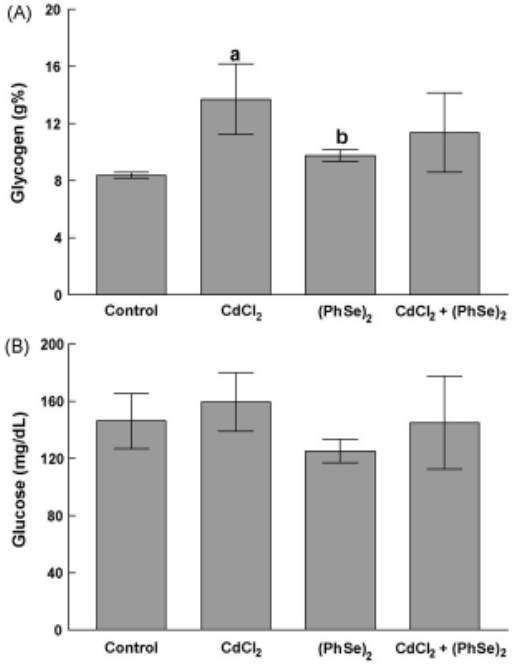

Fig. 5. Effect of $(PhSe)_2$ on hepatic glycogen content (A) and plasma glucose levels (B) of rats exposed to $CdCl_2$. Data are reported as mean ± S.D. of six animals per group. (a) $p < 0.05$ as compared to the control group (two-way ANOVA/Duncan) and (b) $p < 0.05$ as compared to the $CdCl_2$ group (two-way ANOVA/Duncan).

by $CdCl_2$ was abolished by $(PhSe)_2$ administration (Fig. 3C).

3.3. Markers of renal damage

These parameters were evaluated with the aim to determine if $CdCl_2$ and $(PhSe)_2$ exposure changes markers of renal damage. Two-way ANOVA of plasma urea levels revealed a significant $CdCl_2 \times (PhSe)_2$ interaction ($F_{1,32} = 4.64$, $p < 0.039$) (Fig. 4A). Urea levels were increased (23%) in rats exposed with $CdCl_2$. This increase was abolished by $(PhSe)_2$ administration (Fig. 4A).

Two-way ANOVA did not demonstrate significant alterations in plasma creatinine concentration (Fig. 4B).

3.4. Glycogen and glucose concentration

These parameters were evaluated to determine if glucose metabolism was affected by $CdCl_2$ and $(PhSe)_2$ exposure. Two-way ANOVA of hepatic glycogen levels yielded a significant main effect of $CdCl_2$ ($p < 0.05$) (Fig. 5A). Post hoc comparisons demonstrated that rats exposed to $CdCl_2$ presented an increase (64%) in hepatic glycogen levels. $(PhSe)_2$ administration was not effective in ameliorating the hepatic glycogen levels increased by $CdCl_2$.

Two-way ANOVA did not demonstrate significant alterations in plasma glucose concentration (Fig. 5B).

3.5. Oxidative stress

These parameters were carried out with the aim to determine if oxidative stress is affected by $CdCl_2$ and $(PhSe)_2$ exposure.

3.5.1. CAT, SOD, δ-ALA-D and GST activities

Two-way ANOVA did not demonstrate any modification in hepatic δ-ALA-D, CAT, SOD and GST activities (Table 1).

3.5.2. MDA, ascorbic acid, NPSH and MT levels

Two-way ANOVA did not show alteration in MT levels (Table 2). A significant main effect of $(PhSe)_2$ ($p < 0.05$) on hepatic NPSH levels was observed

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Table 1
Effect of $(PhSe)_2$ on δ-ALA-D, CAT, SOD and GST activities in liver of rats exposed to $CdCl_2$

	δ-ALA-D (nmol PBG/(mg protein h))	CAT (U/mg protein)	SOD (U/mg protein)	GST (μmol/(min mg protein))
Control	16.66 ± 0.48	111.21 ± 8.28	28.69 ± 1.40	1.12 ± 0.10
$CdCl_2$	18.17 ± 2.01	109.91 ± 7.92	27.01 ± 5.12	1.19 ± 0.15
$(PhSe)_2$	15.13 ± 2.26	119.96 ± 9.71	27.18 ± 3.23	1.08 ± 0.25
$CdCl_2 + (PhSe)_2$	16.79 ± 1.50	117.30 ± 6.70	26.52 ± 3.21	1.08 ± 0.28

Data are reported as mean ± S.D. from six rats in each group.

Table 2
Effect of $(PhSe)_2$ on MDA, ascorbic acid, NPSH and MT levels in rats exposed to $CdCl_2$

	MDA (pg/mL)	Ascorbic acid (μg/(AA g tissue))	NPSH (μmol/g tissue)	MT (percentage of control)
Control	2.24 ± 0.21	334.57 ± 20.30	19.74 ± 1.14	100 ± 0.81
$CdCl_2$	21.62 ± 1.58[a]	336.70 ± 11.70	19.76 ± 1.86	110.18 ± 13.94
$(PhSe)_2$	1.36 ± 0.18[b]	367.57 ± 16.08[a]	26.26 ± 1.64[a]	95.06 ± 16.21
$CdCl_2 + (PhSe)_2$	2.33 ± 0.24[b]	353.20 ± 17.18	24.31 ± 1.87[a]	99.78 ± 14.02

Data are reported as mean ± S.D. from six rats in each group, except for MDA assay ($n = 3$).
[a] $p < 0.05$ as compared to the control group (two-way ANOVA/Duncan).
[b] $p < 0.05$ as compared to the $CdCl_2$ group (two-way ANOVA/Duncan).

(Table 2). Post hoc comparisons demonstrated that rats exposed to $(PhSe)_2$ presented an increase (33%) in hepatic NPSH levels.

A significant $CdCl_2 \times (PhSe)_2$ interaction in plasma MDA levels was found ($F_{1,8} = 383.46$, $p < 0.001$) (Table 2). Post hoc comparisons demonstrated that rats exposed to $CdCl_2$ presented an increase (about nine fold) in plasma MDA levels. $(PhSe)_2$ administration reduced MDA levels increased by $CdCl_2$ (Table 2).

A significant main effect of $(PhSe)_2$ in hepatic ascorbic acid levels was observed ($p < 0.05$) (Table 2). In fact, rats that received $(PhSe)_2$ presented an increase (33%) in hepatic ascorbic acid levels.

3.6. Cadmium content

Cadmium content in plasma of rats was increased in $CdCl_2$ group when compared to the control group. Administration of $(PhSe)_2$ reduced (about four fold) cadmium content increased by $CdCl_2$ exposure (Table 3).

The increase in hepatic cadmium content induced by $CdCl_2$ exposure was reduced by $(PhSe)_2$ administration (about six fold). Similarly, $(PhSe)_2$ diminished (about two fold) the levels of cadmium in the kidney of rats (Table 3).

3.7. Histopathology

$CdCl_2$ exposure caused a moderate hepatocyte degeneration (ballooning) and discrete necrosis (Fig. 6B) when compared to hepatocytes from the control group (Fig. 6A). Liver of rats exposed to $CdCl_2$ and $(PhSe)_2$ did not have histopathological alteration when compared to the control group (Fig. 6D).

Table 3
Effect of $(PhSe)_2$ on cadmium content (μg/g creatinine) in plasma, liver and kidney of rats exposed to $CdCl_2$

	Plasma	Liver	Kidney
Control	0.5	0.15	0.05
$CdCl_2$	8.33	6.10	2.01
$(PhSe)_2$	0.32	0.10	0.13
$CdCl_2 + (PhSe)_2$	2.15	0.89	1.03

A pool of samples (one pool for each experimental group) was made to evaluate cadmium content ($n = 3$–5 per each group). The assay was made in triplicate to excluded interferences of method.

4. Discussion

Diphenyl diselenide has been shown to protect against toxicity of various chemicals including carcinogens and other inducers of oxidative damage [19–24]. The mechanism by which $(PhSe)_2$ acts has been primarily attributed to inhibition of oxidative stress induced by these chemicals [20,21,24]. The formation of a complex between $(PhSe)_2$ and cadmium has also been reported as a possible mechanism of $(PhSe)_2$ action [24].

The present study demonstrated that co-treatment with $(PhSe)_2$ ameliorated hepatotoxicity and cellular damage in rat liver after sub-chronic exposure with

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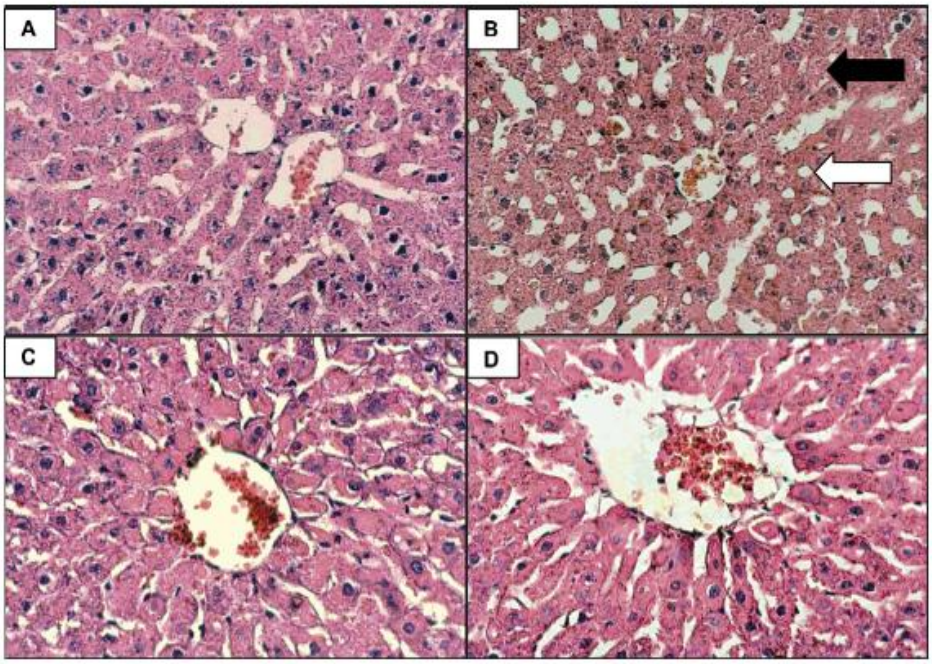

Fig. 6. Liver histopathology. Control group shows normal hepatic architecture (A); $CdCl_2$ group shows ballooning area (white arrow) and necrosis (black arrow) (B); $(PhSe)_2$ group shows normal hepatic architecture (C); $CdCl_2$ and $(PhSe)_2$ group shows hepatic architecture similar to the control group (40×).

$CdCl_2$. The results suggest that $(PhSe)_2$ was effective in ameliorating toxicity induced by $CdCl_2$ by its antioxidant property and by forming a complex with cadmium. The data on cadmium content support the hypothesis that cadmium and $(PhSe)_2$ could be complexed. Based on our data we can suggest that cadmium is more easily excreted in the presence of $(PhSe)_2$, but whether this complex is absorbable or not is an open question.

In this study, $CdCl_2$ exposure increased MDA levels, without any alteration in antioxidant defenses (ascorbic acid levels, CAT and SOD). Thus, it may be possible that oxidative stress was one of the causes for liver damage induced by $CdCl_2$ in this experimental model. Co-treatment with $(PhSe)_2$ abolished the increase in MDA levels induced by $CdCl_2$ exposure. $(PhSe)_2$ is an organoselenium compound that has been reported in view of its antioxidant activity [14]. Accordingly, $(PhSe)_2$ ameliorated oxidative damage induced by $CdCl_2$ in testes of mice [21,23,24].

Conversely, some previous research reports have contested the main role of oxidative stress in cell injury induced by cadmium and other toxicants [43–45]. One possible explanation for the lack of alteration in antioxidant defenses found in this study is the fact that cadmium exposure only alters drastically these defenses in the beginning of exposure [21] but after 30 days of exposure the antioxidant defenses recovered to normal status persisting cellular and oxidative damage. As a result, the cellular and oxidative damage could be reflected on alterations found in biochemical and histopathological parameters.

In addition to oxidative stress, the hepatotoxicity induced by cadmium may involve other three distinct pathways, the first one for the initial injury is caused by the acute direct toxic effects of the metal and/or ischemia due to endothelial cell injury, and the second process for the latter inflammatory injury is one in which Kupffer cell activation and neutrophil infiltration play a major role through triggering a complicated cascade of inflammatory mediators [6,46–49]. The related third process is that cadmium appears to use the transport pathways that also exists for biologically essential metals like calcium, copper and zinc [50,51]. Once inside the cell, cadmium ions can interfere with the cell metabolism mostly by mimicking the action of other divalent cations (especially of calcium) that are employed in activating or inhibiting the action of various enzymes.

In this study sub-chronic exposure with $CdCl_2$ caused liver damage, demonstrated by histopathological alterations. Histopathology evaluation revealed that

Please cite this article in press as: L.P. Borges, et al., Oral administration of diphenyl diselenide protects against cadmium-induced liver damage in rats, Chemico-Biol. Int. (2007), doi:10.1016/j.cbi.2007.09.005

$CdCl_2$ exposure caused a moderate hepatocyte degeneration (ballooning) and a discrete necrosis (Fig. 6B). Co-treatment with $(PhSe)_2$ abolished histopathological alterations induced by $CdCl_2$ (Fig. 6D). Corroborating with the histopathological data, $(PhSe)_2$ reduced the activity of ALT, AST, GGT, ALP and bilirubin levels which are markers of hepatic damage. Moreover, a marker of cellular damage, LDH, also increased after $CdCl_2$ exposure which was ameliorated by $(PhSe)_2$ administration. One hypothesis to explain the beneficial effects of $(PhSe)_2$ in ameliorating biochemical parameters (ALT, AST, GGT, ALP, LDH and bilirubin) is its antioxidant property, scavenging free radicals, preventing lipid peroxidation and consequently the cellular disruption [16]. In fact, previous findings have shown that $(PhSe)_2$ has hepatoprotective properties [19,20,22]. It is worth mentionable that liver is responsible for detoxification of cadmium, but the accumulation of the metal in this organ may cause acute hepatotoxicity [7,52] with histopathological alterations [6].

The results found on cadmium content determination in tissue of rats exposed to $CdCl_2$ provide evidence that the liver is the major cadmium target where $(PhSe)_2$ acts. In fact, the concentration of cadmium in liver was about threefold higher than that in kidney, and $(PhSe)_2$ reduced about sixfold the levels of this metal in liver of rats exposed to $CdCl_2$ (Table 3).

Accordingly, the liver has been reported as the major target organ on repeated administration of cadmium [11], but kidney suffers the effects of biotransformation and subsequent excretion of this metal induces severe nephrotoxicity [5]. Cadmium nephrotoxicity is reversible at early stages and irreversible at advanced stages [4,5]. As mentioned above this study demonstrated that $CdCl_2$ exposure increased urea level but did not alter the creatinine levels. $(PhSe)_2$ ameliorated the renal damage, which was firstly demonstrated by the reduction in cadmium content in this tissue (about twofold reduced) and reflected by a decrease in urea levels. In fact, urea is the first acute renal marker which increases when the kidney suffers any kind of injury otherwise, creatinine is the most trustable renal marker but this increase only occurs when the majority of renal function is lost. The protective effect of $(PhSe)_2$ on renal damage was already reported by Borges et al. [19,20].

In addition, $CdCl_2$ exposure resulted in an increase of liver glycogen content with no alteration in glucose levels. It has been reported that cadmium inhibits the action of Na^+-glucose pump in the gastrointestinal tract and kidneys of mammals [53,54]. The inhibition of the pump probably results in an increased demand for glucose in the animals. This demand could lead to gluconeogenesis, which may explain the normal plasma levels of glucose observed in $CdCl_2$ exposed animals (Fig. 5B). Cadmium also inhibited the activity of glucose-6-phosphate dehydrogenase in several tissues [55–57], leading to an increase in glucose-6-phosphate concentration. The increase in glucose-6-phosphate concentration results in an inactivation of glycogen phosphorylase (inhibition of glycogen degradation) and an activation of glycogen synthase (activation of glycogen synthesis) [58], which possibly explains why $CdCl_2$ exposure increased glycogen content (Fig. 5A). Although, Barbosa et al. [22] have reported that $(PhSe)_2$, in a different experimental protocol, had insulin-like property, $(PhSe)_2$ administration did not alter glycogen or glucose contents.

In conclusion, this study demonstrated that co-treatment with $(PhSe)_2$ ameliorated hepatotoxicity and cellular damage in rat liver after sub-chronic exposure with $CdCl_2$. The proposed mechanisms by which $(PhSe)_2$ acts in this experimental protocol are its antioxidant properties and its capacity to form a complex with cadmium.

Acknowledgements

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

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Please cite this article in press as: L.P. Borges, et al., Oral administration of diphenyl diselenide protects against cadmium-induced liver damage in rats, Chemico-Biol. Int. (2007), doi:10.1016/j.cbi.2007.09.005

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4.3. – A administração oral de disseleneto de difenila potencializa a hepatotoxicidade induzida por tetracloreto de carbono em ratos.

4.3.1. – **Manuscrito 1**

ORAL ADMINISTRATION OF DIPHENYL DISELENIDE POTENTIATES HEPATOTOXICITY INDUCED BY CARBON TETRACHLORIDE IN RATS

Submetido a Hepatology Research

Oral Administration of Diphenyl Diselenide Potentiates Hepatotoxicity Induced by Carbon Tetrachloride in Rats

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Abstract

Carbon tetrachloride (CCl_4) is a model for studying free radical-induced liver injury and screening hepato-protective drugs. Numerous studies have reported the involvement of oxidative stress in CCl_4-induced liver damage and the hepato-protective effects mediated by different antioxidants. The present study examined the effects of diphenyl diselenide, $(PhSe)_2$, on hepatotoxicity induced by CCl_4 in rats. To this end, male Wistar rats received $(PhSe)_2$ by oral route at the dosage of 31.2 mg/kg (dissolved in canola oil) for one or two days. After the second day of treatment, rats received CCl_4 orally (1ml/kg, dissolved in canola oil, 1:1) in a unique dose. The liver and kidney were utilized for determination of histopathology, biochemical [AST (aspartate aminotransferase), ALT (alanine aminotransferase) ALP (alkaline phosphatase), TB (total bilirrubin) and GGT (gamaglutamyl transferase)] and toxicological parameters [thiobarbituric reactive species (TBARS) levels, catalase activity, ascorbic acid, nonprotein thiols (NPSH) and δ-aminolevulinate dehydratase (δ-ALA-D) activity]. Repeated administration of $(PhSe)_2$ caused a marked potentiation of hepatotoxicity induced by CCl_4 exposure, as manifested by an increase in biochemical parameters (AST, ALT, ALP, GGT and BT) and severe alteration in histopathology. This study also demonstrated a potentiation of TBARS levels and a consequent depletion of important antioxidant defenses including catalase and ascorbic acid. This study clearly indicate that $(PhSe)_2$ potentiated acute hepatic damage induced by CCl_4. The oxidative damage is related to the potentiation effect induced by $(PhSe)_2$.

Key-Words: carbon tetrachloride, selenium, diphenyl diselenide, hepatotoxicity, oxidative damage

1. Introduction

Carbon tetrachloride (CCl_4) is a model for studying free radical-induced liver injury and screening hepato-protective drugs (Vitaglione e col., 2004). Through the investigation of acute liver damage induced by CCl_4- in animal models in the past 60 years, it is now generally accepted that CCl_4 toxicity results from its bioactivation to the trichloromethyl free radical by cytochrome P450 isozymes (P450s) (Raucy e col., 1993). The trichloromethyl radical then reacts with oxygen to form the highly toxic reactive trichloromethyl peroxy radical. The free radicals subsequently attack the polyunsaturated fatty acids of membrane lipids to propagate a chain reaction resulting in breakdown of membrane structure, disrupting cell energy processes and protein synthesis (Recknagel e col., 1989; Plaa, 2000; Weber e col., 2003), leading to the progression of liver damage and subsequently inflammation, steatosis, hepatitis, fibrosis, necrosis and hepatocellular carcinoma (Loguercio and Frederico, 2003; Vitaglione e col., 2004).

The liver has unique vascular and metabolic features, which become this organ susceptible to exposition to xenobiotics. Detoxification reactions (phase I and II) metabolize xenobiotics aiming to increase substrate hydrophilicity for excretion. Drug-metabolizing enzymes detoxify many xenobiotics but bioactivate or increase the toxicity of others (Jaeschke e col., 2002). In the case of bioactivation, the liver is the first organ exposed to the dangerous effects of the newly formed toxic compounds (Rao e col., 1997).

Numerous studies have reported the involvement of oxidative stress in CCl_4-induced liver damage and the hepato-protective effects mediated by different

antioxidants (Maxwell e col., 1999; Lee e col., 2002; Ilavarasan e col., 2003). It is interesting to note that antioxidants prevented CCl_4 hepatotoxicity, by inhibiting lipid peroxidation and increasing antioxidant enzyme activities (Teselkin e col., 2000; Kumaravelu e col., 1995). Recently studies have reported that the inhibition of CYP2E1 (which is involved in CCl_4 biotransformation) decreases CCl_4 hepatotoxicity. Otherwise, the induction of this cytochrome increases the drug hepatotoxicity (Weber e col., 2003). Since new pharmaceutical drugs may also be metabolized by CYP enzymes, drug-drug interactions are possible and may affect the safety of using such drugs.

The ability of seleno-organic compounds to reduce hepatoxicity induced by different toxicants has been investigated (Rocha e col., 2005; Barbosa e col., 2006; Borges e col., 2005; 2006). In this context, diphenyl diselenide, $(PhSe)_2$, has demonstrated pharmacological properties including hepato-protective (Borges e col., 2005, 2006), anti-hyperglycemic (Barbosa e col., 2006), anti-inflammatory and antinociceptive (Nogueira e col., 2003; Savegnago e col., 2007), antiulcer (Savegnago e col., 2006), neuroprotective (Nogueira e col., 2004) and antioxidant (Meotti e col., 2004) in different experimental models.

Based on the above considerations, the present study examined the effects of $(PhSe)_2$ on hepatotoxicity induced by CCl_4 in rats.

2. Materials and methods

2.1. Chemicals

Diphenyl diselenide, $(PhSe)_2$, was prepared according to the literature method (Paulmier, 1986) and was dissolved in canola oil. Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed that diphenyl diselenide presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of diphenyl diselenide (99.9%) was determined by GC/HPLC. CCl_4 was obtained from Sigma. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.2. Animals

Male adult Wistar rats (200-250g) and male adult Swiss mice (25-30g) from our own breeding colony were used. The animals were kept in a separate animal room, on a 12 hour light/dark cycle, at a room temperature of 22 $\pm$ 2 ºC and with free access to food and water. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, the Federal University of Santa Maria, Brazil.

2.3. Exposure

To investigate the effects of $(PhSe)_2$ on hepatotoxicity induced by CCl_4 rats received one dose of $(PhSe)_2$ by oral route at dosage of 31.2 mg/kg (1 ml/kg dissolved in canola oil) or two doses of this compound at the same dosage. After the second day of treatment, rats received CCl_4 orally (1mL/kg, dissolved in canola oil, 1:1) in a unique dose (Porchezhian e col., 2005). Each experimental group was constituted of twelve animals.

The dosage of $(PhSe)_2$ was chosen based on its hepatoprotective effect (Borges e col., 2005) and LD_{50} study. The absence of $(PhSe)_2$ group (31.2 mg/kg) is based on the proposal to reduce the number of animals used in laboratories for experimental protocols and supported by data of our research group which demonstrate that this dose did not alter any toxicological parameters in rats (Borges e col., 2005; 2006).

The treatment protocol is given below:

Group Control - canola oil (1 ml/kg) + canola oil + canola oil

Group CCl_4 - canola oil (1 ml/kg) + canola oil + CCl_4 (1 ml/kg)

Group $(PhSe)_2$ - $(PhSe)_2$ (31.2 mg/kg) + $(PhSe)_2$ (31.2 mg/kg) + canola oil (1 ml/kg)

Group $(PhSe)_2$ and CCl_4 - canola oil (1 ml/kg) + $(PhSe)_2$ (31.2 mg/kg) + CCl_4 (1 ml/kg)

Group $(PhSe)_2$ two doses and CCl_4 - $(PhSe)_2$ (31.2 mg/kg) + $(PhSe)_2$ (31.2 mg/kg) + CCl_4 (1 ml/kg)

Twenty four hours after CCl_4 administration, rats were slightly anesthetized for blood collection by heart puncture. An aliquot of 500 μl of total blood was separated on vacuum tubes with heparin. Plasma was obtained by centrifugation at 2,000 x g for 10 min (hemolyzed plasma was discarded) and used for biochemical assays. The liver and kidney were removed and utilized for determination of biochemical and toxicological parameters.

2.4. Biochemical assays

Plasma enzymes AST (aspartate aminotransferase), ALT (alanine aminotransferase) ALP (alkaline phosphatase), BT (total bilirrubin) and γ-GT (γ-

glutamyl transferase) were used as biochemical markers for the early acute hepatic damage and determined by the enzymatic methods of Reitman and Frankel (1957), McComb e col., (1981), Perlman e col., (1974) and Rosalki e col., (1975), respectivelly. Renal function was analyzed by determining plasma urea (Mackay e col., 1927) and creatinine levels (Jaffe e col., 1886).

2.5. Thiobarbituric reactive species (TBARS) levels

Lipid peroxidation in liver and kidney was performed by the formation of TBARS during an acid-heating reaction as previously described by Draper and Hadley (1990). Briefly, the samples were mixed with 1 mL of 10% trichloroacetic acid (TCA) and 1mL of 0.67% thiobarbituric acid (TBA) subsequently they were heated in a boiling water bath for 15 min. TBARS were determined by the absorbance at 532 nm.

2.6. Catalase activity

The liver and kidney were homogenized in 50 mM Tris/HCl, pH 7.5 (1/10, w/v) and centrifuged at 2400×*g* for 15 min. The supernatant was assayed spectrophotometrically by the method of Aebi e col. (1995), which involves monitoring the disappearance of H_2O_2 in the presence of cell homogenate at 240 nm. The enzymatic activity was expressed in Units (1U decomposes 1 μmol H_2O_2/min at pH 7 at 25 °C).

2.7. Ascorbic acid determination

Ascorbic acid determination in liver and kidney was performed as described by Jacques-Silva e col. (2001). 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 1ml 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. Nonprotein thiols (NPSH)

Hepatic and renal NPSH levels were determined by the method of Ellman (1959). The supernatant (500 μL) was mixed (1:1) with 10% trichloroacetic acid (500 μL). After centrifugation, the protein pellet was discarded and free –SH groups were determined in the clear supernatant. An aliquot (100 μL) of supernatant was added in 1M potassium phosphate buffer (850 μL) pH 7.4, and 10 mM 5,5'- dithio-bis(2-nitrobenzoic acid) (DTNB) (50 μL). The colorimetric reaction was measured at 412 nm.

2.9. δ-Aminolevulinate dehydratase (δ-ALA-D) activity

Persuasive evidence has indicated that δ-ALA-D is extremely sensitive to the presence of pro-oxidant agents (Nogueira e col., 2003; Fachinetto e col., 2006), which oxidize –SH groups essential for the enzyme activity (Fernandez-Cuartero e col., 1999). Since this enzyme is very sensitive to xenobiotics, δ-ALA-D activity was used as a marker of toxicity.

δ -ALA-D activity in the liver and kidney 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 *δ* -ALA were used. An aliquot of 200 μL of homogenized tissue was incubated for 1 h (liver) or 2h

(kidney) at 37 °C. The reaction product was determined using modified Erlich's reagent at 555 nm.

2.10. Protein determination

Protein was measured by the method of Lowry e col. (1951) using bovine serum albumin as standard.

2.11. Histopathology

At sacrifice, rats were slightly anesthetized and subjected to a thorough necropsy evaluation. Liver weight was recorded, and tissues were saved and fixed in 10% formalin. For light microscopy examination, tissues were embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin. All groups treated were examined by histopathology.

2.12. Strychnine-induced lethality

The strychnine toxicity test was performed to evaluate whether $(PhSe)_2$ prevents or potentiates lethality as an indicator of effect in cythocrome P450 (Janbaz and Gilani, 2000). Animals were divided into 2 groups (n= 10 mice per group), one group was given the vehicle (canola oil, 1 ml/kg, orally) followed after 30 minutes by the median lethal dose of strychnine (0.6 mg/kg). The animals in group 2 were given similar treatment, except the vehicle was replaced by $(PhSe)_2$ (31.2 mg/kg). The animals were monitored for next 2 hours to count mortalities (Gilani e col., 1996).

2.13. Statistical analysis

Data are expressed as mean ± S.D. Statistical analysis was performed to compare treated groups to respective control groups using 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. Lethality induced by strychnine was statistically analyzed by the χ^2 method and Fisher's exact test.

3. Results

3.1. Biochemical assays

CCl_4 increased AST and ALT activity (about 10-fold) and two doses of $(PhSe)_2$ potentiated the activity of these enzymes. One dose of $(PhSe)_2$ did not alter the increase of ALT but reduced AST activity induced by CCl_4 (Table 1).

Pre-treatment with two doses of $(PhSe)_2$ potentiated the enhance in ALP activity induced by CCl_4 administration. A unique dose of (PhSe) protected against the increase of ALP activity (Table 1).

GGT activity was increased about 3-fold by CCl_4. Repeated doses of $(PhSe)_2$ caused a two-fold increase in GGT activity when compared to CCl_4 group. One dose of $(PhSe)_2$ did not alter GGT activity increased by CCl_4 exposure (Table 1).

Pre-treatment with both dosages of $(PhSe)_2$ potentiated the increase in BT levels induced by CCl_4 (Table 1).

Pre-treatment with two doses of $(PhSe)_2$ increased urea and creatinine levels, which were not altered by CCl_4. A unique dose of $(PhSe)_2$ associated to CCl_4 exposure increased urea levels (Table 1).

3.2. TBARS levels

Both dosages of $(PhSe)_2$ potentiated hepatic TBARS levels induced by CCl_4 (Figure 1A).

Pre-treatment with two doses of $(PhSe)_2$ potentiated renal TBARS level induced by CCl_4. One dose of dose $(PhSe)_2$ protected the augment in renal TBARS levels caused by exposure to CCl_4 (Figure 1B).

3.3. Catalase activity

Repeated dosages of $(PhSe)_2$ potentiated inhibition of catalase activity induced by CCl_4 exposure. A single dose of $(PhSe)_2$ did not alter enzyme activity inhibited by CCl_4 (Figure 2A).

Renal catalase activity was not altered neither by CCl_4 nor by $(PhSe)_2$ dosages (Figure 2B).

3.4. Ascorbic acid determination

Pre-treatment with both dosages of $(PhSe)_2$ potentiated the reduction in ascorbic acid levels in liver (Figure 3A) and kidney (Figure 3B) of rats.

3.5. NPSH levels

CCl_4 exposure did not alter hepatic (Figure 4A) and renal (Figure 4B) NPSH levels. Two doses of $(PhSe)_2$ significantly increased hepatic NPSH levels (Figure 4A).

Renal NPSH levels increased only with one dose of $(PhSe)_2$ and associated to CCl_4 (Figure 4B).

3.6. δ-ALA-D

CCl_4 exposure significantly inhibited δ-ALA-D activity in liver and, both dosages of $(PhSe)_2$ did not alter this effect (Figure 5A).

Two doses of $(PhSe)_2$ inhibited renal δ-ALA-D activity, which was not altered by CCl_4 exposure (Figure 5B).

3.7. Liver histopathology

Histopathology of liver revealed that CCl_4 exposure caused a severe hepatocyte degeneration (ballooning), hepatocyte necrosis and infiltration of leukocytes (Table 2, Figures 6D and 6E) when compared to hepatocytes from liver of control rats (Table 2- Figure 6A). Two doses of $(PhSe)_2$ potentiated the liver damage demonstrated by the severity of histopathological alterations (Table 2, Figures 6H and 6I).

3.8. Strychnine-induced lethality

The treatment with strychnine caused 43% of mortality in mice ($p < 0.036$). The pre-treatment with a single dose of $(PhSe)_2$ prevented the effect of strychnine, abolishing lethality in mice.

4- Discussion

The results of the present study clearly indicate that $(PhSe)_2$ potentiated acute hepatic damage induced by CCl_4. As expected CCl_4 exposure caused a severe liver damage, evidenced by marked alterations in liver histopathology and corroborated by an increase in plasma transaminases. The increase in ALP and

GGT activities indicates that the bile duct was affected in animals exposed to CCl_4. The prejudice of liver functionality was also demonstrated by bilirubin levels increased by CCl_4 exposure.

It has been established that CCl_4 is accumulated in hepatic parenchyma cells and metabolically activated by cytochrome (CYP) P450-dependent monooxygenases to form trichloromethyl radical ($CCl^{\bullet}_3$) (Suja e col., 2004). The bioactivation of CCl_4 is mainly executed by the CYP2E1 isoenzyme, but at higher concentrations CYP2B1, CYP2B2 and CYP3A (only in humans) are capable of attacking this haloalkane (Weber e col., 2003). $CCl^{\bullet}_3$ can also react with oxygen to form its highly reactive derivative trichloromethylperoxy radical ($CCl_3OO^{\bullet}$). Both radicals initiate chain reactions of direct and indirect bond formation with cellular molecules (proteins, nucleic acids, carbohydrates and lipids) impairing crucial cellular processes that may ultimately culminate in extensive peroxidative cell damage and death (Weber e col., 2003; Chan e col., 2005). Therefore, an antioxidant or free radical generation inhibitor is important to protect against CCl_4 induced liver lesions (Castro e col., 1974; Ilavarasan e col., 2003).

Based on the fact that $(PhSe)_2$ has been reported as an antioxidant agent (Meotti e col., 2004) and presented hepatoprotective property against hepatic damage induced by 2-nitropropane (Borges e col., 2005; 2006), we evaluated the effect of this organoselenium compound against hepatotoxicity induced by CCl_4 in rats. Different from our expectation repeated administration of $(PhSe)_2$ caused a marked potentiation of hepatotoxicity induced by CCl_4 exposure, as manifested by an increase in biochemical parameters (AST, ALT, ALP, GGT and BT) and severe alteration in histopathology. Accordingly, this study also demonstrated a potentiation

of TBARS levels, an indirect determinant of lipid peroxidation (Esterbauer, 1996) and a consequent depletion of important antioxidant defenses including catalase and ascorbic acid. The levels of NPSH were not altered in this experimental protocol.

On purpose of explaining whether a potentiation of CCl_4 hepatotoxicity induced by $(PhSe)_2$ was due to enzyme inhibitory or activatory action, the strychnine toxicity test was performed. It has been accepted that inhibitors of CYP P450s can impair the bioactivation of CCl_4 into their respective reactive species and thus provide protection against the prevailing hepatocellular damage (Janbaz and Gilani 2000). Strychnine is a substrate for CYPs and many known inhibitors of CYPs increase the toxicity of strychnine via elevation of plasma level of unmetabolized drug, resulting in potentiation of its effect. The data found on strychnine assay indicate that $(PhSe)_2$ prevented animal death, suggesting an activator action of $(PhSe)_2$ in CYPs. Therefore, one can speculate that the potentiation of CCl_4 hepatotoxicity caused by $(PhSe)_2$ could involve its ability to activate CCl_4 biotransformation. These results are corroborated by Qin and collaborators (2005) which demonstrated that voglibose potentiates the hepatotoxicity of carbon tetrachloride by inducing hepatic CYPs.

Although several isoforms of CYPs 450 may metabolize CCl_4, attention has been focused largely on the CYP2E1 (Raucy e col., 1993). The CYP2E1 protein is localized predominantly in the central zone of the liver lobule (Forkert e col., 1991), which explains the typical centrilobular region of liver damage observed after CCl_4 administration and found in our treatment (view figure 6D, 6E and table 2). After CCl_4 bioactivation, the resulting $CCl_3^{\cdot}$ radical binds covalently to CYP2E1, either to

the active site of the enzyme or to the heme group, thereby causing suicide inactivation (Weber e col., 2003).

Another important finding of this study was the inhibition in hepatic δ-ALA-D activity caused by CCl_4 exposure. To the best of our knowledge, this is the first time that acute exposure to CCl_4 is related to δ-ALA- D inhibition. Accordingly, persuasive evidence has indicated that δ-ALA-D is extremely sensitive to the presence of pro-oxidant agents (Nogueira e col., 2003; Fachinetto e col., 2006), which oxidize –SH groups essential for the enzyme activity (Fernandez-Cuartero e col., 1999; Folmer e col., 2003). Repeated doses of $(PhSe)_2$ did not alter the inhibitory effect of CCl_4 in δ-ALA-D activity.

Several authors have reported that the biotransformation of CCl_4 may produce toxic metabolites to kidney (Comporti, 1985; Miao e col., 1990; Knook e col., 1995; Parola e col., 1992). Therefore, the renal profile was investigated in this study to determine if CCl_4 induces renal damage and/or depletes renal antioxidant defenses. In the current study, CCl_4-exposure did not modify urea and creatinine levels, suggesting that this experimental protocol was not enough to induce renal damage. On the contrary, our data clearly demonstrated that pre-treatment with repeated doses of $(PhSe)_2$ increased urea and creatinine levels, indicating a potentiation effect of this organoselenium compound. One hypothesis to try explain the cited result is that repeated doses of $(PhSe)_2$ activates CCl_4 metabolism. These metabolites could interact with renal biological membranes, increasing oxidative stress which was manifested in this study by increased TBARS levels and depletion of ascorbic acid levels and δ-ALA-D activity.

Different from liver, renal δ- ALA-D activity was not altered by CCl_4 exposure, supporting liver as a major target of this halo-compound in this experimental protocol.

In conclusion, the results of the present study clearly indicate that $(PhSe)_2$ potentiated acute hepatic damage induced by CCl_4. The oxidative damage is related to the potentiation effect induced by $(PhSe)_2$

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

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Tables

Table 1 - Effect of pre-treatment with $(PhSe)_2$ on biochemical parameters of rats

Groups	AST (U/L)	ALT (U/L)	ALP (U/L)	GGT (U/L)	BT (mg/dL)	Urea (mg/dL)	Creatinine (mg/dl)
Control	120± 19	86 ± 16	341 ± 152	7 ± 1.7	0.6± 0.3	41 ±10	0.31 ± 0.13
Se2	91 ± 7	52 ± 14	417 ± 16	8 ± 0.4	0.4 ± 0.2	37 ± 11	0.32 ± 0.07
CCl_4	1603 ± 340*	792± 372*	693 ± 109*	20 ± 5.9*	6.5 ± 4.5*	48 ± 10	0.38 ± 0.11
Se1 +CCl_4	394± 148 [a]	742 ± 89*	473 ± 223 [a]	18 ± 4.0*	11.5 ± 2.2*[a]	55 ± 10*	0.36 ± 0.13
Se2+CCl_4	4437 ± 500*[a]	6600 ± 562*[a]	897 ± 82*[a]	56 ± 11.0*[a]	21.7 ± 4.9*[a]	61 ± 14*[a]	0.95 ± 0.50*[a]

*Denoted $p<0.05$ as compared to the control group (one-way ANOVA/Duncan).
[a] Denoted $p<0.05$ as compared to CCl_4 group (one-way ANOVA/Duncan).
Se1 means one dose of $(PhSe)_2$ 31.2 mg/kg.
Se2 means two doses of $(PhSe)_2$ 31.2 mg/kg.

Table 2- Effect of pre-treatment with $(PhSe)_2$ on CCl_4-induced liver damage in rats

Liver alterations	Groups	Without CCl_4	With CCl_4
Hepatocyte degeneration	Control	0	++++
	Se1	0	+++
	Se2	0	+++++
Hepatocyte necrosis	Control	0	++++
	Se1	0	+++
	Se2	0	+++++
Infiltration of leukocytes (inflammation)	Control	+	++++
	Se1	+	+++
	Se 2	+	+++++

0 -Absent; +-few; ++ -mild; +++ -moderate; ++++ -severe; +++++ -extremely severe

Se1 means one dose of $(PhSe)_2$ 31.2 mg/kg.
Se2 means two doses of $(PhSe)_2$ 31.2 mg/kg.

Legends

Figure 1- Effect of pre-treatment with $(PhSe)_2$ on hepatic (1A) and renal TBARS (1B) levels of rats exposed to CCl_4. Data are reported as mean ± S.D. of twelve animals per group. * Denoted $p<0.05$ as compared to the control group (one-way ANOVA/Duncan). [a] Denoted $p<0.05$ as compared to the CCl_4 group (one-way ANOVA/Duncan). Se1 means one dose of 31.2 mg/kg $(PhSe)_2$. Se2 means two doses of 31.2 mg/kg $(PhSe)_2$.

Figure 2- Effect of pre-treatment with $(PhSe)_2$ on hepatic (2A) and renal catalase (2B) activity of rats exposed to CCl_4. Data are reported as mean ± S.D. of twelve animals per group. * Denoted $p<0.05$ as compared to the control group (one-way ANOVA/Duncan). [a] Denoted $p<0.05$ as compared to the CCl_4 group (one-way ANOVA/Duncan). Se1 means one dose of 31.2 mg/kg $(PhSe)_2$. Se2 means two doses of 31.2 mg/kg $(PhSe)_2$.

Figure 3- Effect of pre-treatment with $(PhSe)_2$ on hepatic (3A) and renal (3B) ascorbic acid levels of rats exposed to CCl_4. Data are reported as mean ± S.D. of twelve animals per group. * Denoted $p<0.05$ as compared to the control group (one-way ANOVA/Duncan). [a] Denoted $p<0.05$ as compared to the CCl_4 group (one-way ANOVA/Duncan). Se1 means one dose of 31.2 mg/kg $(PhSe)_2$. Se2 means two doses of 31.2 mg/kg $(PhSe)_2$.

Figure 4- Effect of pre-treatment with $(PhSe)_2$ on hepatic (4A) and renal (4B) NPSH levels of rats exposed to CCl_4. Data are reported as mean ± S.D. of twelve animals per group. * Denoted $p<0.05$ as compared to the control group (one-way ANOVA/Duncan). Se1 means one dose of 31.2 mg/kg $(PhSe)_2$. Se2 means two doses of 31.2 mg/kg $(PhSe)_2$.

Figure 5- Effect of pre-treatment with $(PhSe)_2$ on hepatic (5A) and renal (5B) δ-ALA-D activity of rats exposed to CCl_4. Data are reported as mean ± S.D. of twelve animals per group. * Denoted $p<0.05$ as compared to the control group (one-way ANOVA/Duncan). Se1 means one dose of 31.2 mg/kg $(PhSe)_2$. Se2 means two doses of 31.2 mg/kg $(PhSe)_2$.

Figure 6- Histology of control liver (6A), one dose of 31.2 mg/kg $(PhSe)_2$ (6B), two doses of 31.2 mg/kg $(PhSe)_2$ (6C), CCl_4 -treated group (6D, E), CCl_4 plus one dose of 31.2 mg/kg $(PhSe)_2$ (6F, 6G), CCl_4 plus two doses of 31.2 mg/kg $(PhSe)_2$ (6H, 6I). The black arrow demonstrated necrotic areas and the white arrow, balooning cels.

Figures

Figure 1A

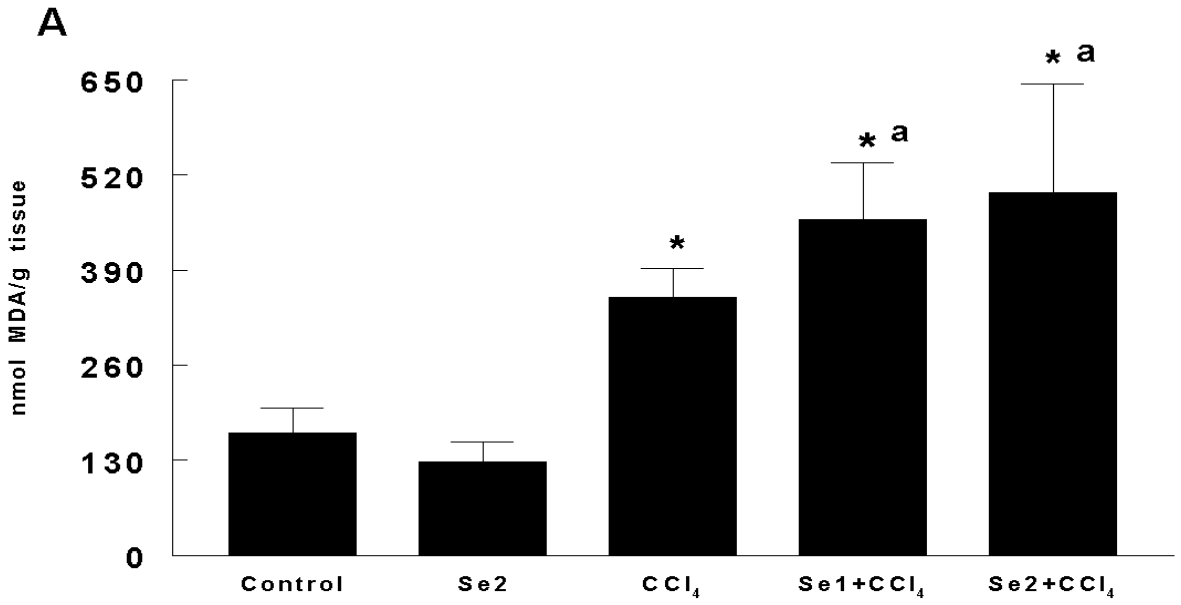

Figure 1B

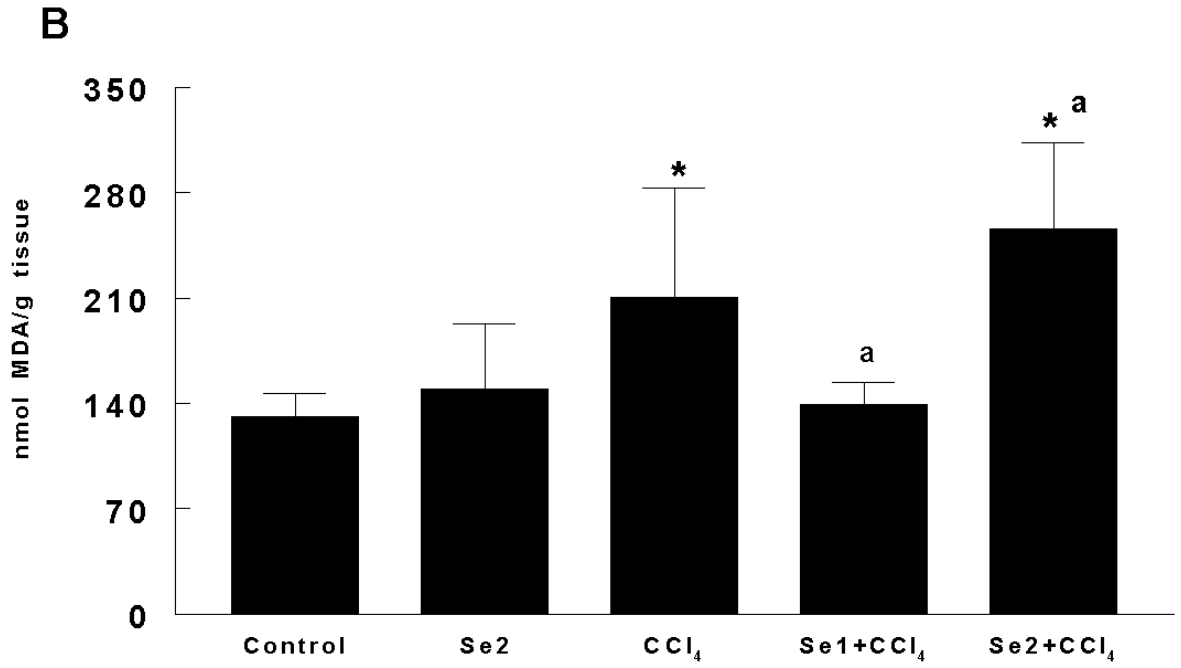

Figure 2A

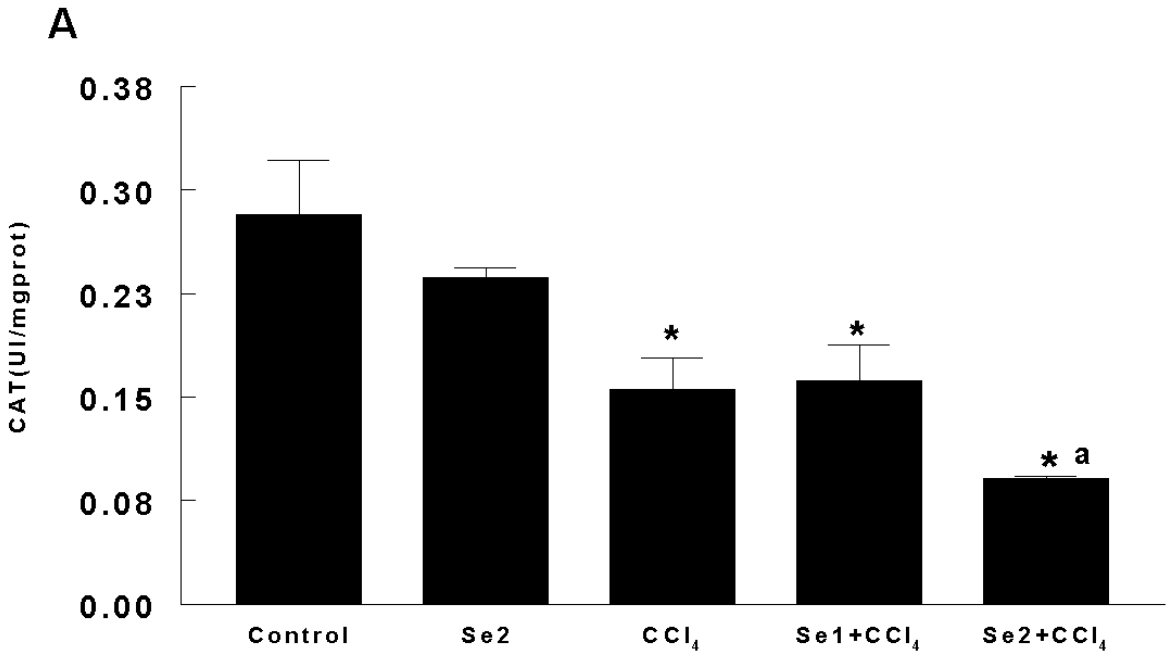

Figure 2B

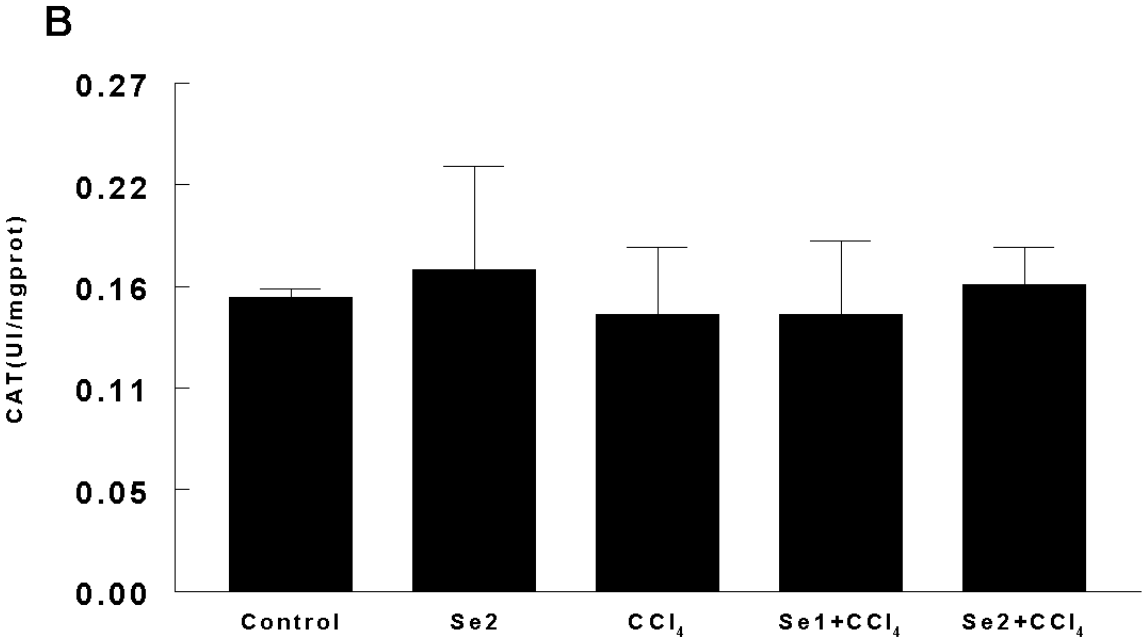

Figure 3A

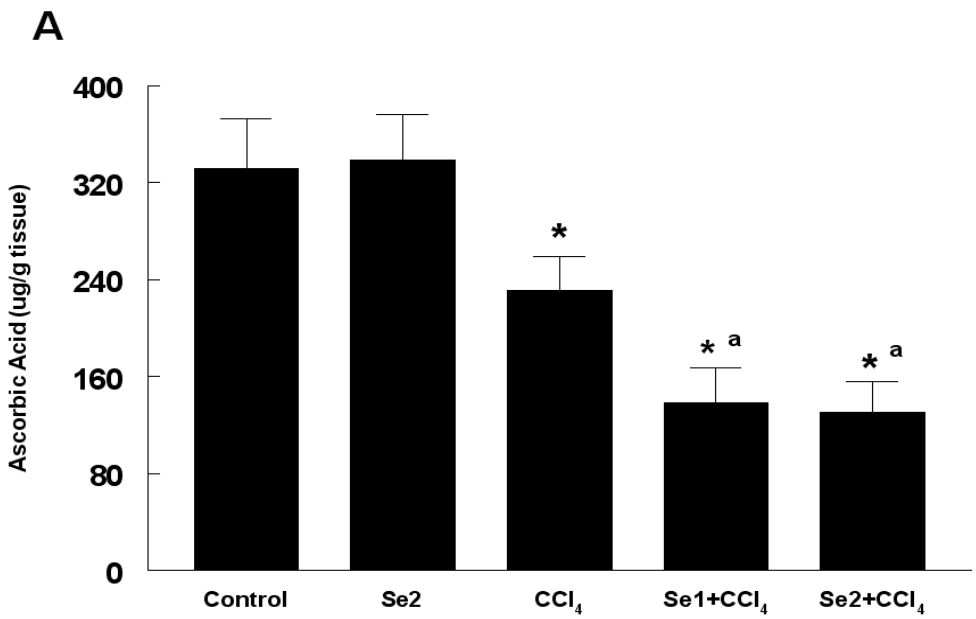

Figure 3B

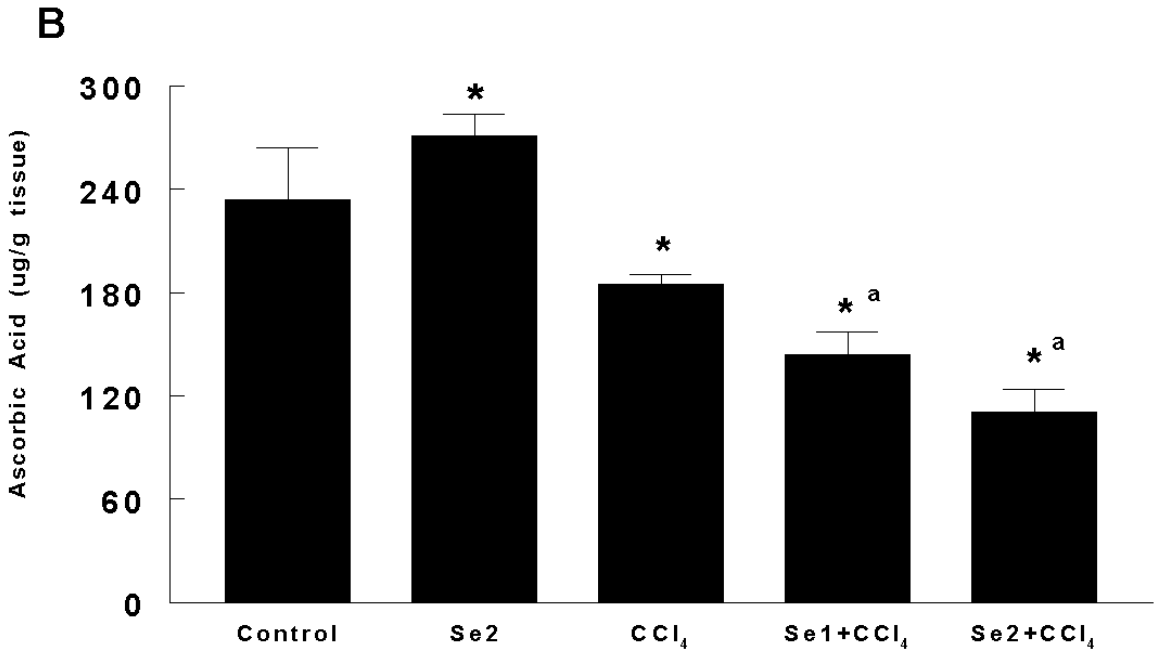

Figure 4A

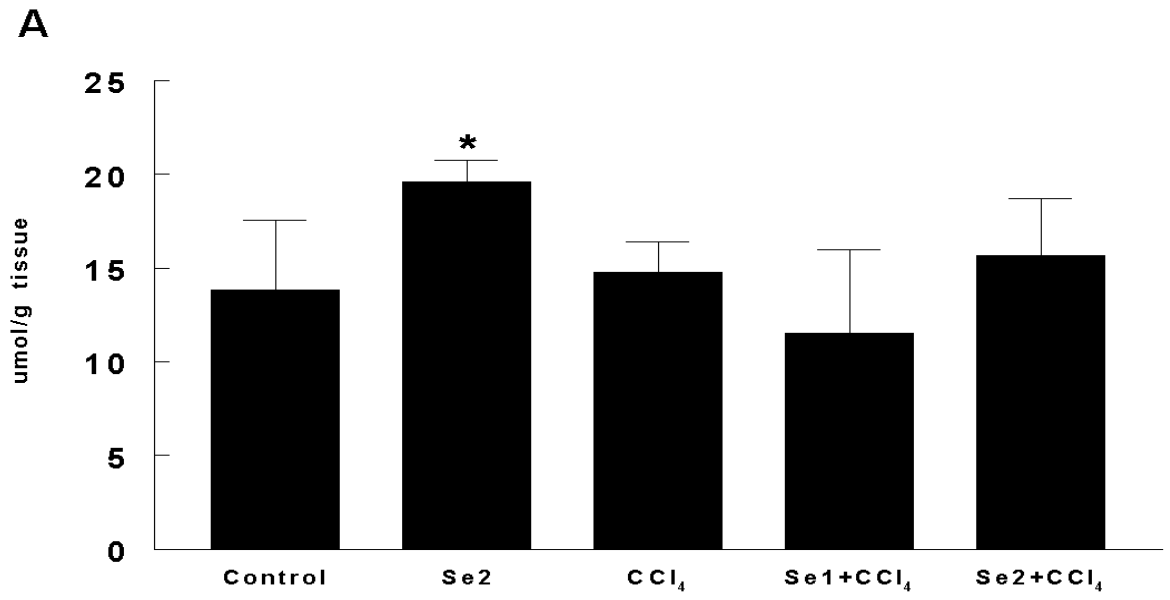

Figure 4B

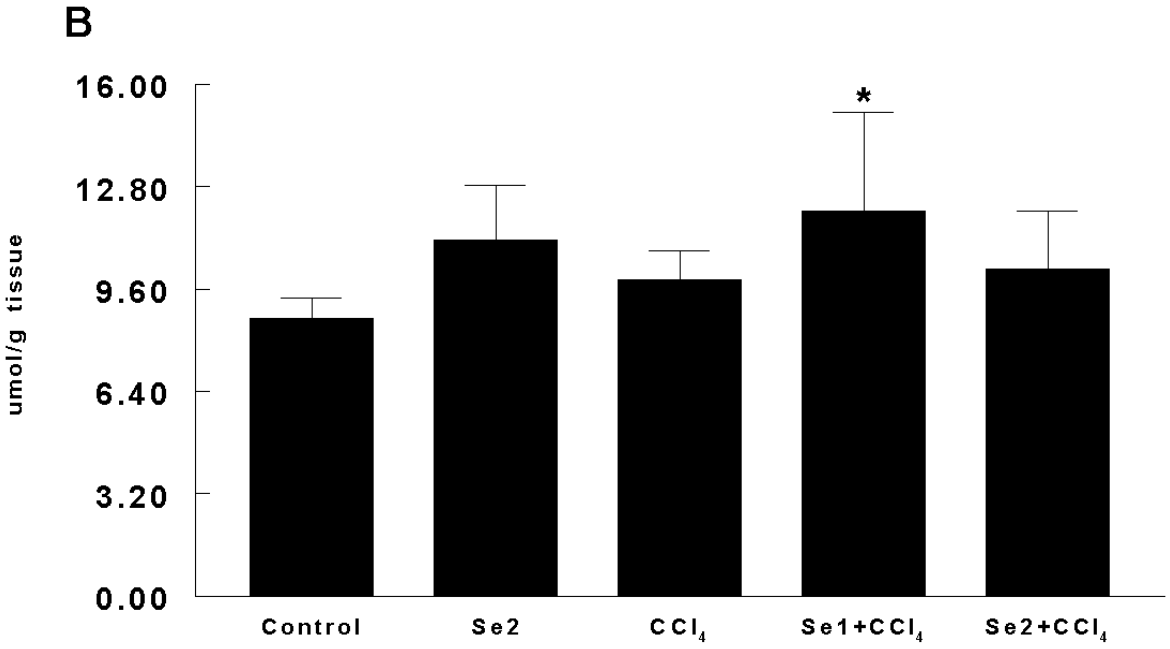

Figure 5A

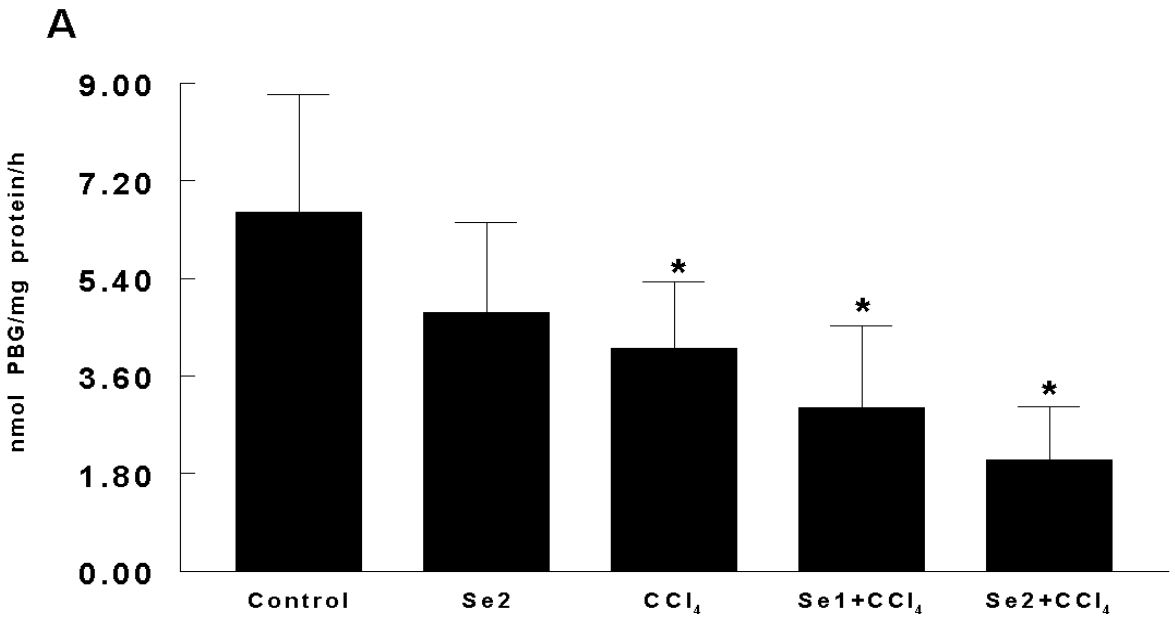
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Figure 5B

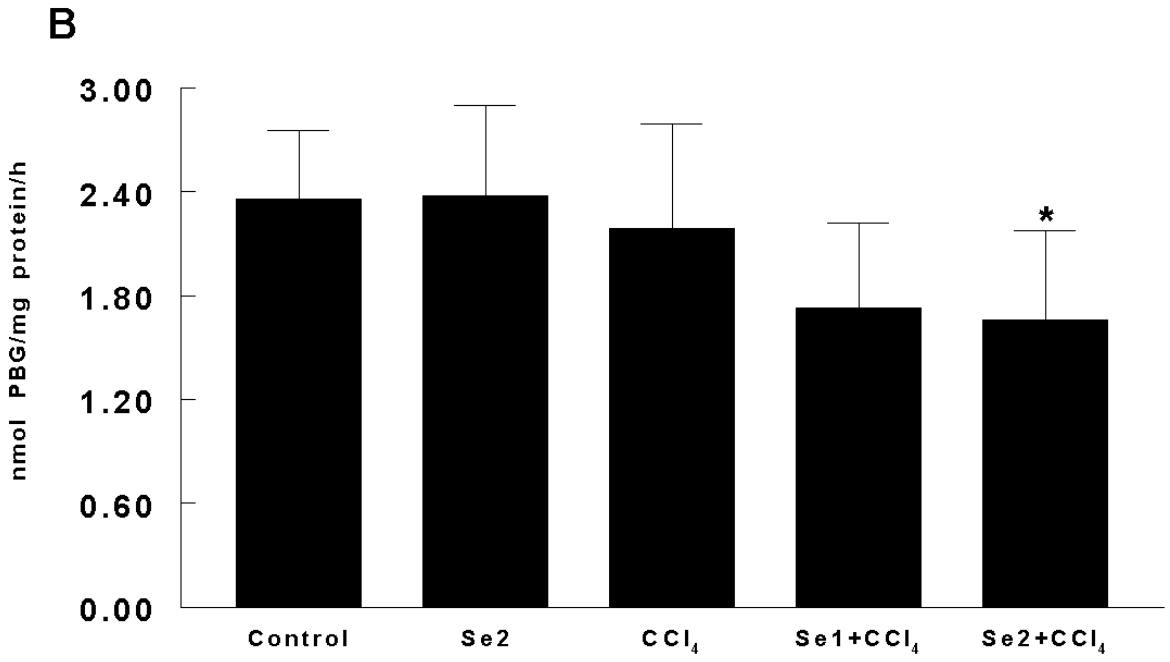
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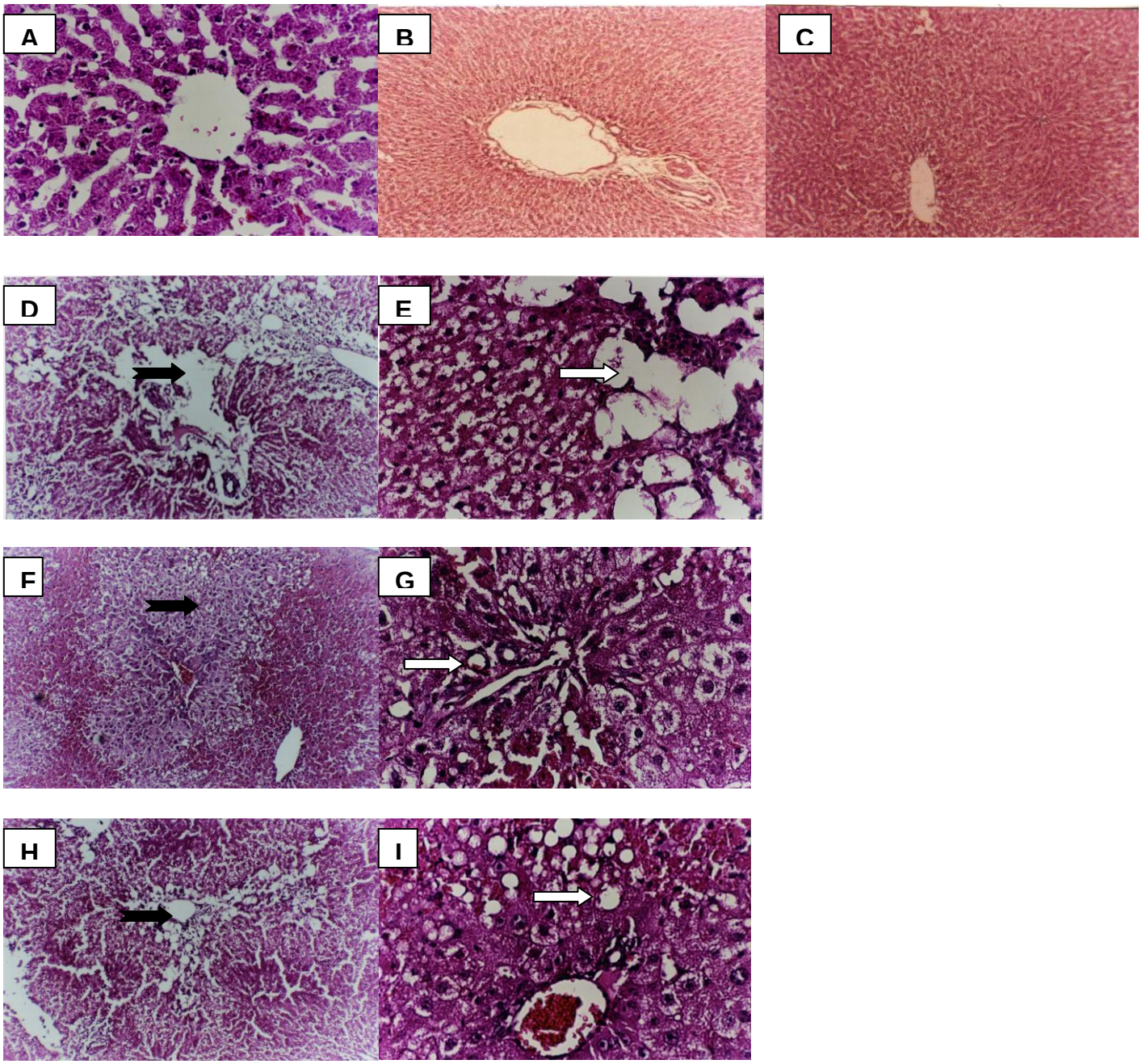
Figure 6



4.4.- Dano hepático agudo induzido por 2-nitropropano em ratos: Efeito causado pelo disseleneto de difenila sobre as defesas antioxidantes

4.4.1- Artigo 3

ACUTE LIVER DAMAGE INDUCED BY 2-NITROPROPANE IN RATS: EFFECT OF DIPHENYL DISELENIDE ON ANTIOXIDANT DEFENSES

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Chemico-Biological Interactions

Available online at www.sciencedirect.com
SCIENCE DIRECT
Chemico-Biological Interactions 160 (2006) 99–107
Chemico-Biological Interactions
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Acute liver damage induced by 2-nitropropane in rats: Effect of diphenyl diselenide on antioxidant defenses

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Received 23 September 2005; received in revised form 17 December 2005; accepted 20 December 2005
Available online 30 January 2006

Abstract

The effect of post-treatment with diphenyl diselenide on liver damage induced by 2-nitropropane (2-NP) was examined in male rats. Rats were pre-treated with a single dose of 2-NP (100 mg/kg body weight dissolved in canola oil). Afterward, the animals were post-treated with a dose of diphenyl diselenide (10, 50 or 100 μmol/kg). The parameters that indicate tissue damage such as liver histopathology, plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transferase (GGT), urea and creatinine were determined. Since the liver damage induced by 2-NP is related to oxidative damage, lipid peroxidation, superoxide dismutase (SOD), catalase (CAT) and ascorbic acid level were also evaluated. Diphenyl diselenide (50 and 100 μmol/kg) effectively restored the increase of ALT and AST activities and urea level when compared to the 2-NP group. At the higher dose, diphenyl diselenide decreased GGT activity. Treatment with diphenyl diselenide, at all doses, effectively ameliorated the increase of hepatic and renal lipid peroxidation when compared to 2-NP group. 2-NP reduced CAT activity and neither alter SOD activity nor ascorbic acid level. This study points out the involvement of CAT activity in 2-NP-induced acute liver damage and suggests that the post-treatment with diphenyl diselenide was effective in restoring the hepatic damage induced by 2-NP.

Keywords: 2-Nitropropane; Organoselenium; Hepatic damage; Antioxidant; Catalase; SOD; Ascorbic acid

1. Introduction

Selenium is an essential micronutrient for both animals and humans as an integral component of several enzymes with antioxidant properties, including glutathione peroxidase and phospholipid hydrogen glutathione peroxidase [1–3].

The advances in the area of synthesis and reactivity of organoselenium compounds, as well as, the discovery that selenium is an essential trace element in the diet [4] has prompted intense studies on the biological properties of organic selenium compounds [5,6]. The selenium atom participates, as a component, in mammalian thioredoxin system, which has been shown to directly reduce lipid hydroperoxides and play a specific role in peroxynitrite defense [7]. In fact, recent study has reported that diaryl diselenides are potent antioxidant compounds [8], among them diphenyl diselenide presented the highest thiol peroxidase activity and antioxidant potential [9]. The discovery of the pharmacological potential of

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doi:10.1016/j.cbi.2005.12.010

organoselenium compounds [9–11] increases the necessity of explanations about the mechanisms of action on cellular levels, considering the oxidative stress as a focus on the discussion. Indeed, the antioxidant activity could explain some protective effects of diphenyl diselenide on oxidative models of damage [12–14].

In general, reactive oxygen species (ROS) generated by any cellular damage play a pivotal role in accelerating oxidative stress in biological systems [15,16]. Lipid peroxidation, an index of oxidative stress, is known to be stimulated in stressed tissues after single administration with a hepatocarcinogen, which subsequently manifests in serious pathological problems [17].

2-Nitropropane, a nitroalkane, is used as a constituent of paints and inks, in the manufacture of chemicals as industrial solvent and can be found in cigarette smoke [18]. 2-NP has been found to cause hepatotoxicity in occupationally exposed humans [19,20] and in rats and rabbits [21,22]. The mechanism by which 2-NP exerts hepatotoxicity is not clearly understood, but many authors suggested that 2-NP metabolism may increase ROS levels and cause cellular damage [15,23,24]. Thus, liver damage is a therapeutic target of selenorganic compounds, as well as, the various clinical conditions in which hydroperoxides play a role.

The cellular environment has some antioxidative enzymes and non-enzymatic mechanisms (Vitamin C, reduced glutathione) to counteract the damaging effects of reactive oxygen species generated after a single administration of 2-NP [16,25]. Catalase and peroxidases are the primary antioxidant defenses against the increase of free radicals [16]. Similarly, superoxide dismutase (SOD), another enzymatic antioxidant defense, can readily react with damaging superoxide and OH radicals and convert them into less reactive radicals.

Taking these in consideration, the aim of the present study was to investigate the effect of diphenyl diselenide on toxicological parameters and evaluate the role of some antioxidant defenses (enzymatic and non-enzymatic) to counteract the damaging effects of ROS generated after administration of 2-NP in rats.

2. Material and methods

2.1. Chemicals

Diphenyl diselenide (Fig. 1) was synthesized according to literature methods [26] and was dissolved in canola oil. Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed that diphenyl diselenide presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of diphenyl diselenide (99.9%) was determined by GC/HPLC. 2-Nitropropane (2-NP) was obtained from Sigma. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

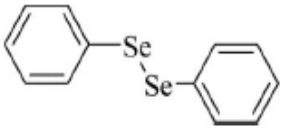


Fig. 1. Chemical structure of diphenyl diselenide.

2.2. Animals

Male adult albino Wistar rats (200–250 g) from our own breeding colony were used. The animals were kept in separate animal rooms, on a 12-h light/12-h dark cycle, at a room temperature of 22 ± 2 °C and with free access to food and water. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, University Federal of Santa Maria, Brazil.

2.3. Exposure

Six animals per group were usually tested in the experiments. Rats were injected intraperitoneally with a single dose of 2-NP (100 mg/kg body weight dissolved in canola oil) (groups 2, 6, 7 and 8) according to Fiala et al. [23]. Twenty-four hours later, animals were injected with diphenyl diselenide (10, 50 or 100 μmol/kg, i.p.) (groups 3, 4, 5, 6, 7 and 8). The control group received only vehicle (canola oil, 5 mL/kg) (group 1). All groups were sacrificed 48 h after 2-NP injection. Rats were slightly anesthetized with chloroformio for blood collection by heart puncture (hemolyzed serum was discarded). The liver and kidney were dissected and frozen on ice-cold until the time of assay.

The protocol of rat treatments is given below:

Group (1) Canola oil (i.p.) plus canola oil (5 mL/kg, i.p.).
Group (2) 2-NP (100 mg/kg, i.p.) plus canola oil (5 mL/kg, i.p.).
Group (3) Canola oil (5 mL/kg, i.p.) plus diphenyl diselenide (10 μmol/kg, i.p.).
Group (4) Canola oil (5 mL/kg, i.p.) plus diphenyl diselenide (50 μmol/kg, i.p.).
Group (5) Canola oil (5 mL/kg, i.p.) plus diphenyl diselenide (100 μmol/kg, i.p.).
Group (6) 2-NP (100 mg/kg, i.p.) plus diphenyl diselenide (10 μmol/kg, i.p.).

Group (7) 2-NP (100 mg/kg, i.p.) plus diphenyl diselenide (50 μmol/kg, i.p.).

Group (8) 2-NP (100 mg/kg, i.p.) plus diphenyl diselenide (100 μmol/kg, i.p.).

2.4. Renal profile

Renal function was analyzed using a commercial Kit (LABTEST, Diagnostica S.A., Minas Gerais, Brazil) by determining plasma urea [27] and creatinine [28].

2.5. Hepatic profile

Plasma enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT) and γ-glutamyl transferase (γ-GT) were used as the biochemical markers for the early acute hepatic damage [29], using a commercial kit (LABTEST, Diagnostica S.A., Minas Gerais, Brazil).

2.6. Antioxidant defenses

2.6.1. Superoxide dismutase activity

Superoxide dismutase activity in liver was assayed spectrophotometrically as described by Misra and Fridovich [30]. 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.

2.6.2. Catalase activity

The liver was homogenized in 50 mM Tris–HCl, pH 7.5 (1/10, w/v) and centrifuged at 2400 × *g* for 15 min. The supernatant was assayed spectrophotometrically by the method of Aebi et al. [31], which involves monitoring the disappearance of H_2O_2 in the presence of cell homogenate at 240 nm. The enzymatic activity was expressed in Units (1 U decomposes 1 μmol H_2O_2/min at pH 7 at 25 °C).

2.6.3. Ascorbic acid determination

Ascorbic acid determination was performed as described by Jacques-Silva et al. [32]. Liver protein was precipitated in 10 vol. of 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 containing 4.5 mg/mL dinitrophenyl hydrazine and $CuSO_4$ (0.075 mg/mL).

2.7. Lipid peroxidation

Lipid peroxidation was performed by the formation of thiobarbituric acid reactive species (TBARS) during an acid-heating reaction as previously described by Drapur and Hodley [33]. Briefly, the samples were mixed with 1 mL of 10% TCA and 1 mL of 0.67% thiobarbituric acid subsequently they were heated in a boiling water bath for 15 min. TBARS were determined by the absorbance at 535 nm and were expressed as nmol MDA/g wet tissue.

2.8. Histopathology

At sacrifice, rats were anesthetized and subjected to a thorough necropsy evaluation. Organ weight for liver was recorded, and tissues were saved and fixed in 10% formalin. For light microscopy examination, tissues were embedded in paraffin sectioned at 5 μm and stained with hematoxylin and eosin.

2.9. Statistical analysis

Data were analyzed by analysis of variance (ANOVA) (2-nitropropane × diphenyl diselenide), followed by Duncan's multiple-range test when appropriate. Differences between groups were considered significant when $p < 0.05$. Main effects are presented only when the higher (second) order interaction was non-significant.

3. Results

3.1. Renal profile

Two-way ANOVA of urea level yielded a significant 2-NP × $(PhSe)_2$ interaction. Post hoc comparisons demonstrated that 2-NP increased ($p < 0.05$) urea level when compared to the control group. Diphenyl diselenide at 10 μmol/kg is not capable of restoring the increase of urea level induced by 2-NP (Table 1). Post-treatment with 50 and 100 μmol/kg diphenyl diselenide significantly restores the urea level when compared to the 2-NP group (Table 1).

Creatinine levels were not altered in all treated groups (data not shown).

3.2. Hepatic profile

Two-way ANOVA of ALT activity yielded a significant 2-NP × $(PhSe)_2$ interaction. Post hoc comparisons demonstrated that 2-NP increased ALT activity (2.3-fold higher than the control group) ($p < 0.05$) and the post-treatment with diphenyl diselenide (50 and

Table 1
Effect of post-treatment with diphenyl diselenide on toxicological parameters in 2-NP-induced liver damage in rats

Groups	Urea (mg/dL)	ALT (U/L)	AST (U/L)	GGT (U/L)	Ascorbic acid[a]
Control	43 ± 5.3	37 ± 3.0	171 ± 24	3.7 ± 0.9	181 ± 20
Se 10[#]	47 ± 7.4[b]	36 ± 6.0[b]	196 ± 10[b]	4.1 ± 0.7[b]	211 ± 39
Se 50	46 ± 5.2[b]	32 ± 4.0[b]	192 ± 10[b]	3.6 ± 0.5[b]	205 ± 27
Se 100	39 ± 7.1[b]	33 ± 4.0[b]	145 ± 4.0[b]	4.0 ± 0.6[b]	201 ± 25
2-NP	66 ± 12[*]	84 ± 12[*]	227 ± 61[*]	14 ± 1.4[*]	194 ± 32
2-NP + Se 10	62 ± 6.0[*]	90 ± 6.0[*]	253 ± 46[*]	14 ± 1.2[*]	184 ± 30
2-NP + Se 50	45 ± 7.5[b]	42 ± 4.1[b]	197 ± 15[b]	8.0 ± 2.2[*]	213 ± 39
2-NP + Se 100	39 ± 13[b]	38 ± 6.0[b]	125 ± 19[b]	3.7 ± 0.9[b]	138 ± 26[*,b]

Data are expressed as mean ± S.D. of six animals per group.
[a] Data of hepatic ascorbic acid level are presented as μg ascorbic acid/g wet tissue.
[b] Denoted $p < 0.05$ as compared to 2-NP group (ANOVA/Duncan).
[*] Denoted $p < 0.05$ as compared to the control group (ANOVA/Duncan).
[#] Selenium doses are presented as μmol/kg.

100 μmol/kg) was effective in reducing ALT activity. The lower dose of diphenyl diselenide did not restore ALT activity (Table 1).

Two-way ANOVA of AST activity yielded a significant 2-NP × $(PhSe)_2$ interaction. Post hoc comparisons demonstrated that 2-NP increased AST activity (1.3-fold higher than the control group) ($p < 0.05$). Diphenyl diselenide at 50 and 100 μmol/kg could restore the increment in AST activity induced by 2-NP administration. The lower dose of diphenyl diselenide did not alter the AST activity (Table 1).

Two-way ANOVA of GGT activity yielded a significant 2-NP × $(PhSe)_2$ interaction. Post hoc comparisons demonstrated that 2-NP changed GGT activity (3.8-fold higher than the control group) ($p < 0.05$). Post-treatment with diphenyl diselenide (100 μmol/kg) was effective in restoring the increase in GGT activity, while 50 μmol/kg $(PhSe)_2$-treatment partially restored GGT activity induced by 2-NP. The lower dose of diphenyl diselenide did not modify GGT activity induced by 2-NP (Table 1).

3.3. Catalase activity

Two-way ANOVA of CAT activity yielded a significant 2-NP × $(PhSe)_2$ interaction. Post hoc comparisons demonstrated that 2-NP reduced CAT activity ($p < 0.05$). The highest dose of diphenyl diselenide was effective in restoring catalase activity. Post-treatment with 10 and 50 μmol/kg diphenyl diselenide was not effective in restoring enzyme activity when compared to the control group. When given alone, diphenyl diselenide at 10 and 50 μmol/kg, but not at the highest dose, reduced CAT activity (Fig. 2).

3.4. Superoxide dismutase activity

A significant main effect of diphenyl diselenide was shown by two-way ANOVA of SOD activity ($p < 0.05$). Post hoc comparisons indicated that 100 μmol/kg diphenyl diselenide given alone and diphenyl diselenide (100 μmol/kg) plus 2-NP were associated with a significant increase of about 1-fold in SOD activity ($p < 0.05$). SOD activity remained unchanged in rats treated with 2-NP and diphenyl diselenide, individually or after combined treatment with lower and intermediate doses (Fig. 3).

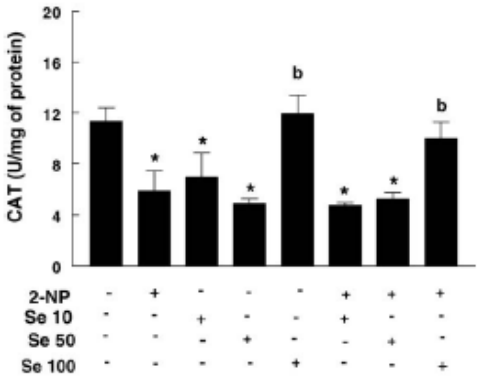


Fig. 2. Effect of diphenyl diselenide and 2-NP or their combination on catalase activity in liver of 2-NP-exposed rats. The catalase activity was expressed as U/mg of protein. Data are reported as mean ± S.E.M. of six animals per group. [*]Denoted $p < 0.05$ as compared to the control group (two-way ANOVA/Duncan). [b]Denoted $p < 0.05$ as compared to the 2-NP group (ANOVA/Duncan).

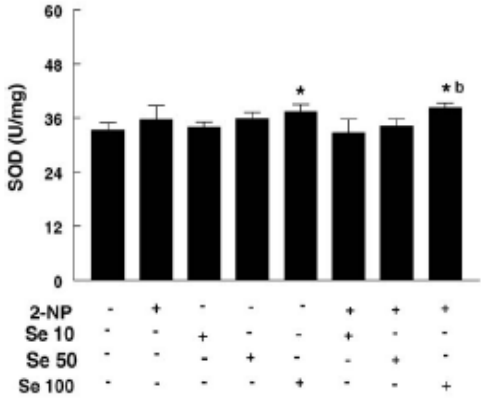


Fig. 3. Effect of diphenyl diselenide and 2-NP or their combination on superoxide dismutase activity in liver of 2-NP-exposed rats. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinefrine autoxidation by 50% at 26 °C. Data are reported as mean ± S.E.M. of six animals per group. *Denoted $p < 0.05$ as compared to the control group (two-way ANOVA/Duncan). bDenoted $p < 0.05$ as compared to the 2-NP group (ANOVA/Duncan).

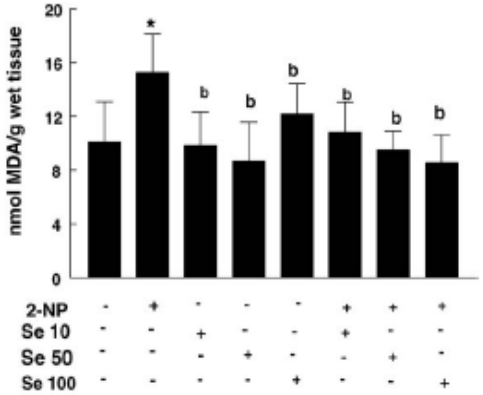


Fig. 4. Effect of diphenyl diselenide and 2-NP or their combination on TBARS levels in liver of 2-NP-exposed rats. Data are reported as mean ± S.E.M. of six animals per group. *Denoted $p < 0.05$ as compared to the control group (two-way ANOVA/Duncan). bDenoted $p < 0.05$ as compared to the 2-NP group (ANOVA/Duncan).

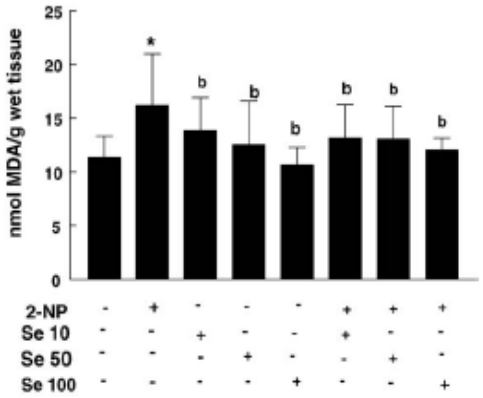


Fig. 5. Effect of diphenyl diselenide and 2-NP or their combination on TBARS levels in kidney of 2-NP-exposed rats. Data are reported as mean ± S.E.M. of six animals per group. *Denoted $p < 0.05$ as compared to the control group (two-way ANOVA/Duncan). bDenoted $p < 0.05$ as compared to the 2-NP group (ANOVA/Duncan).

3.5. Ascorbic acid determination

Two-way ANOVA of ascorbic acid content yielded a significant 2-NP × $(PhSe)_2$ interaction. Post hoc comparisons demonstrated that treatment with 2-NP plus 100 μmol/kg $(PhSe)_2$ significantly reduced (23.7%) ascorbic acid levels ($p < 0.05$) (Table 1). Ascorbic acid levels remained unchanged in 2-NP and all other groups (Table 1).

3.6. Lipid peroxidation

Two-way ANOVA of TBARS levels yielded a significant 2-NP × diphenyl diselenide interaction. Post hoc comparisons demonstrated that 2-NP increased ($p < 0.05$) lipid peroxidation in liver (50% higher than the corresponding control group) (Fig. 4). Diphenyl diselenide, at all doses tested, was effective in restoring the increase in TBARS levels (Fig. 4).

2-NP increased renal TBARS levels (43% higher than the corresponding control group) (Fig. 5). Post-treatment with $(PhSe)_2$, at all doses, was effective in completely restoring the augment in renal TBARS levels (Fig. 5).

3.7. Histopathology

Histopathological examination of liver revealed that 2-NP-treatment causes a severe swelling (Fig. 6C), lymphocytic infiltration (Fig. 6E) and confluent necrosis on centro-lobular zone (Fig. 6D) when compared to the control group (Fig. 6A). Degenerative changes were not evident on groups 5 (Fig. 6B) and 8 (Fig. 6F and G).

4. Discussion

Intraperitoneal administration of 2-NP in rats caused a marked increase in toxicological parameters and decrease in CAT activity. It is well known that 2-NP is an excellent acute model to induce hepatic damage [21,23,34–36]. The hepatotoxicity induced by 2-NP is not completely understood, but the liver has been implicated as the principal site of 2-NP metabolism [37],

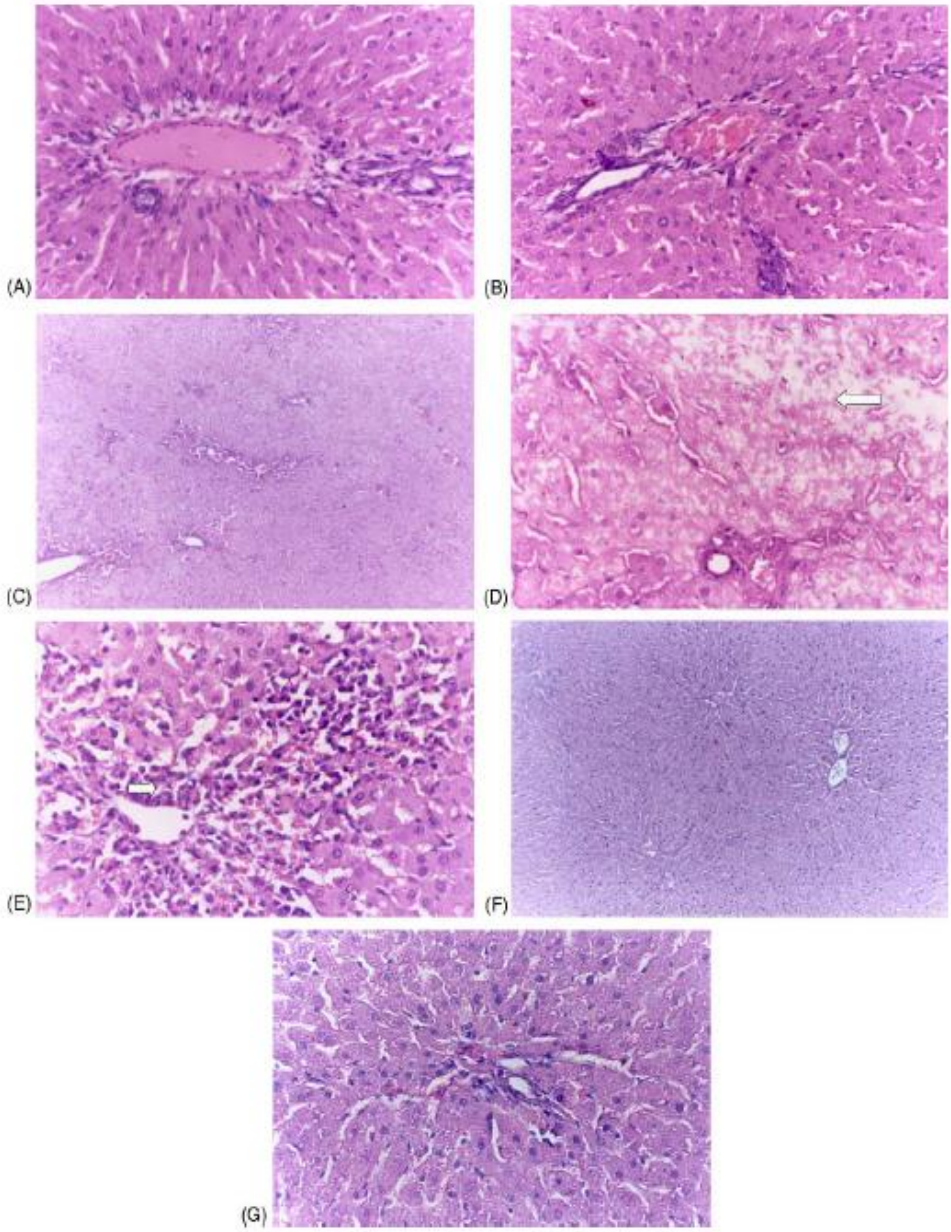


Fig. 6. Histological evaluation of liver from control animals (40×) (A) and from 100 μmol/kg diphenyl diselenide group (40×) (B). Observe a severe swelling in hepatocytes (20×) (C), confluent necrosis on centro-lobular zone (arrow) (40×) (D) and lymphatic inflammation infiltred on portal space (arrow) (40×) (E) in 2-NP (100 mg/kg) group, and the absence of alterations in liver from 2-NP (100 mg/kg) plus 100 μmol/kg diphenyl diselenide group (F (20×) and G (40×)).

which generates acetone and nitrite by the microsomal cytochrome P-450 system [38], NO radicals [15] and increases the peroxidative damage [39], which probably induces the hepatic lesion.

Accordingly, the present study demonstrated an increase in hepatic lipid peroxidation levels and an inhibition of CAT activity after 2-NP-treatment. The post-treatment with diphenyl diselenide at all doses tested was

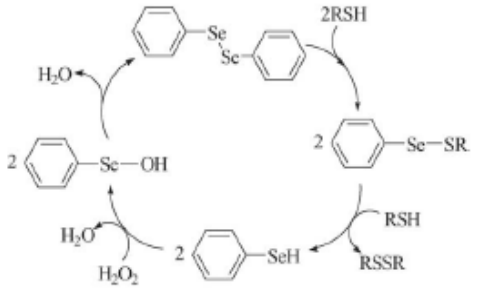
effective in reducing lipid peroxidation status toward the normal level. In fact, 2-NP inhibited CAT activity and diphenyl diselenide was able to restore the enzyme activity suggesting that 2-NP can promote lipid peroxidation by directly inhibiting CAT activity. Another interesting observation in the present study is that 2-NP did not induce lipid peroxidation by a direct effect on SOD activity. In addition, diphenyl diselenide at the highest dose increased SOD activity and reduced ascorbic acid level demonstrating a direct effect on these parameters. This study also demonstrated that ascorbic acid level was not altered after 2-NP exposure. Thus, the treatment with the highest dose of diphenyl diselenide ameliorates the hepatic TBARS level by increasing CAT activity for the purpose of restoring the increment in lipid peroxidation induced by 2-NP. The decrease in catalase activity at 10 and 50 μmol/kg of $(PhSe)_2$ is paradoxical and may indicate a complex interaction between the antioxidant properties of the selenium compound and a decrease in an important antioxidant enzyme in liver. In spite of this, the administration of these doses of diphenyl diselenide was not accompanied by sign of oxidative stress in the liver. In line with this, the plasma activities of ALT and AST, classical markers of hepatic toxicity, were not modified by these doses of $(PhSe)_2$. For the case of 2-NP injected rats, the decrease in hepatic catalase activity could have contributed to the increase in TBARS levels and to the pathological changes observed in the liver. Taken together, these results indicate that a lowered CAT activity cannot explain alone the toxicity of 2-NP and other factors that are associated with its decrease, for example, lipid peroxidation also plays a role in determining its final hepatic toxicity.

The administration of 2-NP induces an increase in AST, ALT and GGT activities. The animals post-treated with diphenyl diselenide in the doses of 50 and 100 μmol/kg can restore the increment in ALT and AST activities induced by 2-NP. However, when GGT activity was measured only 100 μmol/kg diphenyl diselenide was effective in completely restoring the increase of GGT activity. The data obtained from hepatic markers indicate that diphenyl diselenide was able to restore the liver damage induced by 2-NP. It is interesting to note the differential sensitivity of the liver enzyme markers to the protective effect of diphenyl diselenide. In fact, ALT and AST activities were restored with a lower dose of diphenyl diselenide than GGT. Gamma-glutamyltransferase is produced by the liver cell microsomes and is widely distributed in cells that are involved in the secretion and absorption of bile. In contrast, ALT and AST have a distinct subcellular distribution and their differential sensitivity to diphenyl deselenide treatment, when compared to GGT, may be related to the fact that diselenide restores first the overall cell function and will interact with microsomes only after high doses. Alternatively, the differential sensitivity of the markers may be related to the fact that the microsomal fraction is the primary target for 2-NP. Consequently, a higher dose of diselenide was required to restore the enzyme marker associate with it. Consistent with these findings, recently, our group has reported a hepato-protective effect of diphenyl diselenide in diabetic rats [14] and in acute liver damage induced by 2-NP [12].

In addition, 2-NP increased renal TBARS level and the post-treatment with diphenyl diselenide at all doses tested was effective in reducing TBARS status toward the control level. The nitroalkane also increased urea level and the treatment with diphenyl diselenide (50 and 100 μmol/kg) was effective in restoring the increase in renal markers. Accordingly, Kim et al. [40] have reported that 2-NP is a potent nephrotoxin and this compound induced substantial rises in LPO products in kidney. Conversely, recent findings reported by Borges et al. [12] demonstrated that 2-NP did not alter renal TBARS, it could be explained by the fact that animals treated with 2-NP have been sacrificed 24 h after exposure, suggesting that renal toxicity is time-dependent. In this study, 2-NP animals were sacrificed 48 h after nitroalkane exposure. Therefore, in this work it is supposed that liver is the main target organ for 2-NP metabolism [37] and after that, kidney is the responsible to excrete 2-NP metabolites [40] suffering the toxicity effect on renal cells demonstrated by an increase in renal TBARS level. We speculated that compared to the liver, the amount of free radicals produced by 2-NP metabolism exceeds antioxidant enzyme activities, and thus kidney function is disrupted and tissue damage developed. Another interesting observation of this study is that diphenyl diselenide effects do not tally across the doses used, the lack of dose–response effect is in accordance with a previous study published by us [12].

Similar to the mechanism proposed by Morgenstern et al. [41], we believe that diphenyl diselenide restores 2-NP-induced damage, at least in part, via the generation of phenyl selenol species from the reaction between thiol groups and diphenyl diselenide, which involves one electron transfer. Thus, a new covalent Se–S bond is formed in which each atom (Se and S) donated one electron to construct this bond, as shown in Scheme 1.

The results obtained in this study are corroborated by recent studies reporting a large number of pharmacological properties presented by diphenyl diselenide [9–14,42]. It is noteworthy that diphenyl diselenide, at doses similar or upper to those used in this study, was



Scheme 1.

safe to rodents and did not alter renal and hepatic markers [43].

In conclusion, the results detailed here show that the inhibition of CAT activity may be related to LPO development in the liver from 2-NP treated rats. Moreover, SOD and ascorbic acid possibly did not influence the mechanism of action of 2-NP-induced acute liver damage. Collectively, the present data have revealed that diphenyl diselenide, when injected intraperitoneally in rats, acts in the antioxidant defenses increasing SOD and CAT activities.

Acknowledgements

The financial support by FAPERGS, CAPES and CNPq is gratefully acknowledged. J.B.T.R., C.W.N. and G.Z. are the recipients of CNPq fellowships.

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5- DISCUSSÃO

O uso do disseleneto de difenila em diversos protocolos experimentais despertou o crescente interesse no estudo de suas propriedades farmacológicas e seu mecanismo de ação ainda pouco esclarecido. De fato, este organocalcogênio possui algumas atividades já descritas tais como: efeitos anti-úlcera (Savegnago e col., 2006), antiinflamatório e antinociceptivo (Nogueira e col., 2003c; Ghislene e col., 2003; Zasso e col., 2005), anti-hiperglicemiante (Barbosa e col., 2006), protege contra a discinesia orofacial induzida por reserpina e haloperidol (Burger e col., 2004, 2006), pode atuar na facilitação da formação de memória em camundongos (Rosa e col., 2006) e possui propriedades antioxidantes em diversos modelos experimentais (Rossato e col., 2002; Meotti e col., 2004; Santos e col., 2004; 2005; Posser e col., 2006).

Entretanto, existem poucos estudos na literatura que descrevam sobre o possível papel hepatoprotetor deste composto. Devido a isso, torna-se importante o desenvolvimento de estudos sobre os efeitos e mecanismos de ação do $(PhSe)_2$ frente a agentes hepatotóxicos.

Baseando-se nestas perspectivas, um dos nossos objetivos foi avaliar o efeito do $(PhSe)_2$ perante o modelo de indução de dano hepático pela administração de 2-NP. Os resultados obtidos no **Artigo 1** indicam que o $(PhSe)_2$ protegeu contra o dano hepático agudo induzido por 2-NP. De fato, estudos demonstraram que compostos orgânicos de selênio podem atuar como agentes terapêuticos no tratamento do dano hepático (Nogueira e col., 2004).

O $(PhSe)_2$ na dose de 100 μmol/kg protegeu contra o aumento dos marcadores hepáticos (ALT, γ-GT e α-fetoproteína), renais e de estresse oxidativo

induzido pela administração única de 2-NP. Barbosa e colaboradores (2006) também demonstraram que o $(PhSe)_2$ possui propriedade hepatoprotetora em ratos com diabetes, corroborando com os resultados obtidos no nosso estudo. Considerando a hepatotoxicidade do 2-NP, este nitroalcano também induziu alterações macro e microscópicas avaliadas por inspeções visuais e histopatológicas, respectivamente. De fato, estudos revelaram que as alterações no microambiente celular com destruição da integridade do hepatócito e liberação do conteúdo intracelular para a circulação, antecedem as alterações histopatológicas (Prasada e Hariharaq, 1991; Machle e col., 1940).

As avaliações histopatológicas demonstraram desde áreas com edema moderado até zonas com alterações degenerativas nos hepatócitos, dados estes corroborados por Zitting e colaboradores (1981). Interessantemente a administração de $(PhSe)_2$ (10, 50 e 100 μmol/kg) protegeu de maneira dependente da dose, a incidência do dano hepático induzido por 2-NP.

Os mecanismos pelos quais o $(PhSe)_2$ exerce sua propriedade hepatoprotetora não estão totalmente bem descritos. Entretanto, sabe-se que a administração de compostos de selênio reduz o estresse oxidativo (Meotti e col., 2004; Santos e col., 2004; 2005; Posser e col., 2006), possivelmente por inativar os efeitos dos intermediários nocivos oriundos do metabolismo do 2-NP, incluindo óxidos de nitrogênio e seus radicais (Kohl e col., 1995).

Paralelo ao estudo anteriormente descrito, investigou-se o potencial curativo do $(PhSe)_2$ através do modelo de reversão do dano hepático induzido por 2-NP. Os resultados obtidos no **Artigo 3** indicam que a administração i.p. de $(PhSe)_2$ reverte o dano hepático induzido pela administração de 2-NP.

De fato, a administração de 2-NP causou um aumento nos marcadores hepáticos, alterações histopatológicas e uma inibição na atividade da catalase hepática com subseqüente elevação da peroxidação lipídica. Contudo, parece que o mecanismo de indução de dano hepático causado por 2-NP não envolve alterações na atividade da superóxido dismutase (SOD) e nos níveis de ácido ascórbico. A administração de $(PhSe)_2$ possivelmente age nas defesas antioxidantes, principalmente sobre a atividade da CAT, resultando na reversão do dano hepático.

Similar aos resultados propostos por Morgenstern e colaboradores (1992), acredita-se que o disseleneto de difenila restaura o dano hepático induzido por 2-NP, pelo menos em parte, pela geração de espécies de fenil-selenol a partir da reação de grupos tióis e o $(PhSe)_2$, o qual envolve a transferência de um elétron para a formação de uma nova ligação covalente (Se-S) (esquema 1-**Artigo 3**).

Além dos nitroalcanos extrapolamos nosso protocolo experimental, investigando o dano hepático induzido por metais pesados, dos quais escolhemos o cádmio. Este metal pesado é amplamente utilizado em diversos processos industriais e está presente na fumaça do cigarro, assim como o 2-NP, e a exposição ao Cd pode induzir lesões graves em diversos tecidos (Santos e col., 2004; 2005). Sendo assim, o objetivo deste trabalho foi estudar os efeitos do $(PhSe)_2$ sobre a indução de dano hepático, induzido pelo $CdCl_2$ em ratos.

Os resultados obtidos no **Artigo 2** sugerem que o tratamento concomitante com o $(PhSe)_2$ evita o dano celular e hepático induzidos pela administração sub-crônica de $CdCl_2$. Estes resultados são corroborados por outros autores que utilizaram o $(PhSe)_2$ em modelos de indução de estresse oxidativo pela administração de Cd (Santos e col., 2004; 2005a; 2005b).

O modelo de indução de dano hepático pela administração de $CdCl_2$ é caracterizado por mecanismos de ações paralelas que culminam com o dano hepático. Sabe-se que o Cd pode interagir diretamente com as células endoteliais privando-as de oxigenação ou também pelo seu potencial pró-inflamatório (Kuester e col., 2002; Mousa, 2004; Zhao e col., 2006) e ainda por ser mimético de cátions divalentes, como o cálcio, interferindo em enzimas importantes ao metabolismo (Baker e col., 2003; Fotakis, 2006).

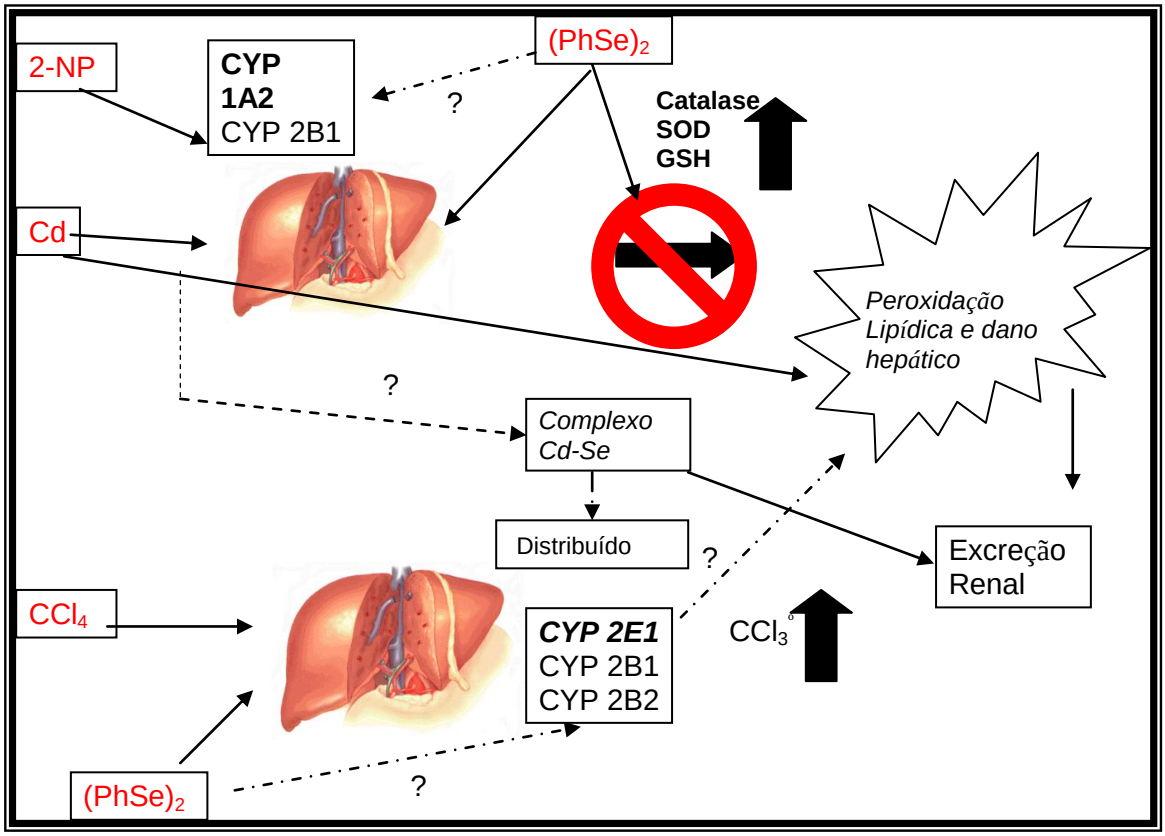
Baseando no descrito acima, o mecanismo de ação do $(PhSe)_2$ frente a indução de dano por Cd, possivelmente pode estar relacionado com sua propriedade antioxidante e sua capacidade de formação de um complexo com o Cd, impedindo as ações tóxicas deste metal. Tais hipóteses já foram confirmadas parcialmente em estudos desenvolvidos pelo nosso grupo (Santos e col., 2004; 2005). Entretanto, a questão se o complexo Cd-Se formado é distribuído ou não deve ser investigada nos próximos estudos.

Embora os resultados obtidos nos **Artigos 1 e 2** sejam promissores, seguindo os objetivos deste estudo, foi utilizado um terceiro modelo experimental de dano hepático induzido pela administração aguda de CCl_4, composto muito conhecido pela sua toxicidade e com mecanismo de ação bem descrito na literatura. Surpreendentemente os resultados obtidos no **Manuscrito 1** indicaram que o $(PhSe)_2$, nas doses utilizadas no estudo, potencializava o dano hepático agudo induzido pela administração de CCl_4 e o dano oxidativo poderia estar relacionado com este efeito.

Na tentativa de elucidar os mecanismos de potencialização do dano hepático observado neste estudo, tentamos explorar indiretamente o mecanismo de

biotransformação do CCl_4 pelo sistema citocromo P-450 e a possível interação do $(PhSe)_2$ na metabolização deste hepatotoxicante. Para isso, foi utilizado o modelo de interação do $(PhSe)_2$ com a estriquinina, onde evidenciou-se que o $(PhSe)_2$ age como indutor da CYPs, aumentando indiretamente o potencial tóxico do CCl_4. Este dado obtido no **manuscrito 1** fortalece a hipótese que o $(PhSe)_2$ possa ser um indutor do sistema P-450, provavelmente o CYP2E1, já que estudos demonstraram que drogas com importante ação hepatoprotetora inibem a biotransformação do CCl_4 pelo CYP2E1. Qin e colaboradores (2005) também demonstraram que o extrato de voglibose (com ação antioxidante) potencializou o dano hepático induzido por CCl_4 por indução do sistema P-450. Vale ressaltar que ensaios de quantificação dos diferentes tipos de CYPs, mediante a administração de CCl_4 e $(PhSe)_2$ são fundamentais para determinar exatamente qual das CYPs são ativadas ou inibidas neste protocolo experimental.

Portanto, devido aos resultados obtidos neste estudo podemos inferir que o $(PhSe)_2$ possui potencial hepatoprotetor nos modelos de indução de dano hepático pela administração de 2-NP e Cd (**Artigos 1 e 2**). Este efeito pode ser extrapolado quando o dano já está estabelecido, demonstrando também as propriedades curativas deste composto (**Artigo 3**). Entretanto, parece que a interação do $(PhSe)_2$ com o sistema de biotransformação P-450 pode potencializar os efeitos do dano hepático induzido pelo CCl_4 (**Manuscrito 1**), sugerindo que o $(PhSe)_2$ pode interagir com o sistema P-450, mas os subtipos específicos de CYP onde o $(PhSe)_2$ pode ser biotransformado ainda permanecem obscuros na literatura.



Esquema 1: Representação esquemática dos possíveis mecanismos de ação do disseleneto de difenila. As setas contínuas indicam os dados observados neste estudo e as setas pontilhadas, as possíveis hipóteses.

6- CONCLUSÕES

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

- O disseleneto de difenila administrado i.p. conseguiu proteger contra o dano hepático induzido por 2-NP, possivelmente devido à propriedade antioxidante deste composto, observado pela diminuição dos níveis de peroxidação lipídica.
- No modelo de indução de dano hepático pela adminstração oral de Cd, o disseleneto de difenila foi capaz de proteger contra este dano possivelmente devido a sua propriedade antioxidante e pela formação de um complexo com o Cd.
- O disseleneto de difenila administrado por via oral potencializa o dano hepático induzido pela administração oral de CCl_4, provavelmente devido a interação do $(PhSe)_2$ com o sistema de biotransformação P-450, o qual está embasado nos resultados obtidos no teste com estricnina.
- O disseleneto de difenila possui potencial curativo no modelo de indução de dano pela administração i.p. de 2-NP, sendo o aumento das defesas antioxidantes os possíveis responsáveis pelo efeito terapêutico deste organocalcogênio.

7- PERSPECTIVAS

Tendo em vista os resultados obtidos a respeito do papel hepatoprotetor do disseleneto de difenila. Ainda, é necessário avaliar os mecanismos de biotransformação deste composto. Dessa forma, poderíamos realizar este estudo a partir da concretização dos seguintes objetivos:

- Identificar os possíveis sistemas de citocromos envolvidos na metabolização do disseleneto de difenila.
- Investigar o efeito deste composto na reversão do dano hepático induzido por CCl_4 e Cd.
- Estudar o efeito deste composto em outros modelos de indução de dano hepático.
- Comparar o efeito do disseleneto de difenila com hepatoprotetores já consagrados.

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