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Tese de Doutorado

DISSELENETO DE DIFENILA: UM ORGANOCALCOGÊNIO COM BAIXA TOXICIDADE EM NÃO ROEDORES E IMPORTANTES PROPRIEDADES ANTI-ATEROGÊNICAS EM MODELOS *IN VIVO* E *IN VITRO*

Andreza Fabro de Bem

Santa Maria, RS, Brasil

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DISSELENETO DE DIFENILA: UM ORGANOCALCOGÊNIO COM BAIXA TOXICIDADE EM NÃO ROEDORES E IMPORTANTES PROPRIEDADES ANTI-ATEROGÊNICAS EM MODELOS *IN VIVO* E *IN VITRO*

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por

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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**.

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Elaborada por **Andreza Fabro de Bem** como requisito parcial para a obtenção do grau de **Doutor em Bioquímica Toxicológica**

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“A mente que se abre a uma nova idéia
jamais voltará ao seu tamanho original”

Albert Einstein

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LISTA DE ABREVIATURAS

ALT: alanina aminotransferase

ANOVA: análise de variância

Apo B: apoproteína B

AST: aspartato aminotransferase

δ-ALA-D: δ-ácido aminolevulínico desidratase

BAEC: células endoteliais de aorta bovina

CAT: catalase

ERN: espécies reativas de nitrogênio

ERO: espécies reativas de oxigênio

GPx: glutationa peroxidase

GSH: glutationa reduzida

LDL: lipoproteína de baixa densidade

MCP-1: proteína quimiotática de monócitos

M-CSF: fator de diferenciação e proliferação de monócitos

mmLDL: LDL minimamente oxidada

$^{\bullet}$NO: óxido nítrico

$O_2^{\bullet-}$: superóxido

$ONOO^-$: peroxinitrito

$(PhSe)_2$: disseleneto de difenila

PUFA: ácido graxo poliinsaturado

Se: Selênio

SHNP: tióis não protéicos

TBARS: espécies reativas ao ácido tiobarbitúrico

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Bioquímica Toxicológica

Universidade Federal de Santa Maria, RS, Brasil

Disseleneto de difenila: Um organocalcogênio com baixa toxicidade em não roedores e importantes propriedades anti-aterogênicas em modelos *in vivo* e *in vitro*

AUTORA: Andreza Fabro de Bem

ORIENTADOR: João Batista Teixeira da Rocha

DATA E LOCAL DA DEFESA: Santa Maria, junho de 2007.

A aterosclerose é uma condição inflamatória crônica associada à produção de espécies oxidantes. A oxidação da lipoproteína de baixa densidade (LDL) parece ter um papel fundamental na patogênese da aterosclerose, desta forma os antioxidantes, substâncias capazes de inibir o processo oxidativo, podem ser úteis na prevenção das condições patológicas associadas à aterosclerose, como doenças cardiovasculares e acidentes vasculares cerebrais. Nesta tese, investigou-se os potenciais efeitos toxicológicos e as propriedades anti-aterogênicas do disseleneto de difenila $(PhSe)_2$, um simples composto orgânico de selênio que apresenta importantes propriedades antioxidantes. Os estudos toxicológicos revelaram que o $(PhSe)_2$ oralmente administrado produziu poucos efeitos tóxicos nos coelhos expostos ao composto por um longo período de tempo. O principal efeito tóxico do $(PhSe)_2$ foi a diminuição dos níveis de ácido ascórbico no sangue, fígado e cérebro dos coelhos. Todavia, a exposição prolongada ao $(PhSe)_2$ não alterou os marcadores clássicos de lesão hepática e renal e os demais marcadores do estresse oxidativo. O consumo de $(PhSe)_2$ por coelhos hipercolesterolêmicos diminuiu significativamente os níveis sorológicos de colesterol total e ainda reduziu os níveis sanguíneos e hepáticos de TBARS, bem como a produção de espécies oxidativas no sangue e no cérebro destes animais. Estudos *in vitro* demonstraram que o $(PhSe)_2$ protegeu LDLs humanas isoladas contra a oxidação induzida por Cu^{2+} e AAPH e a oxidação do ácido parinárico (PnA) incorporado nas LDLs de maneira concentração dependente. O $(PhSe)_2$ também foi capaz de prevenir a oxidação da porção protéica das LDLs. Estes efeitos protetores do $(PhSe)_2$ foram associados a sua atividade tiol peroxidase. Em experimentos utilizando cultura primária de células endoteliais de aorta bovina (BAEC), evidenciou-se o efeito protetor do $(PhSe)_2$ contra a morte celular induzida pelo peroxinitrito, de forma mais efetiva que o ebselen. Os níveis intracelulares de glutationa (GSH) foram completamente consumidos pelo peroxinitrito. O $(PhSe)_2$ *per se* aumentou significativamente os níveis de GSH de maneira concentração dependente, todavia, a adição dos compostos não preveniu o consumo de GSH pelo peroxinitrito. Este efeito pode ser relacionado com o significativo aumento na atividade celular da glutationa peroxidase (GPx) promovido pelo $(PhSe)_2$. Em conclusão estes resultados indicam que a suplementação oral com $(PhSe)_2$ demonstrou baixa toxicidade nos coelhos mesmo após uma ingestão prolongada do composto e sugere-se um novo papel para o $(PhSe)_2$ como um potencial agente anti-aterogênico.

Palavras-chave: Aterosclerose, disseleneto de difenila, glutationa peroxidase, hipercolesterolemia, peroxinitrito, coelhos, toxicidade.

ABSTRACT

Thesis of Doctor's Degree

Federal University of Santa Maria, RS, Brazil

Diphenyl Diselenide: an organoselenium compound with low toxicity in non rodents and importants antiatheorgenic properties

AUTHOR: Andreza Fabro de Bem

ADVISOR: João Batista Teixeira da Rocha

DATE AND PLACE OF THE DEFENSE: Santa Maria, June 2007

Atherosclerosis is a chronic inflammatory condition associated with an overproduction of oxidant species. Since low density lipoprotein (LDL) oxidation plays a key role in the pathogenesis of atherosclerosis, antioxidants that can inhibit this oxidative process might be useful in preventing atherosclerosis-related pathological conditions, such as cardiovascular diseases and stroke. Here, we investigated the potential toxicological effects and the anti-atherogenic properties of diphenyl diselenide $(PhSe)_2$, a simple organoselenium compound that presents important antioxidant properties. Toxicological studies, revealed that $(PhSe)_2$, orally administered, produced minor toxicological effects in rabbits exposure for a long term. The toxic potential of $(PhSe)_2$ was evidenced by a decrease in the ascorbic acid contents in blood, liver and brain of rabbits. Nevertheless, the prolonged exposition with $(PhSe)_2$ was not hepato or renotoxic and not modified the others oxidative stress parameters. The consumption of $(PhSe)_2$ by hipercholesterolemic rabbits significantly decreased the serum cholesterol levels and also diminished the serum and hepatic levels of TBARS, as well as the production of free radical species in blood and brain. *In vitro* studies demonstrated that $(PhSe)_2$ showed a dose-dependent protective effect against Cu^{2+} and 2,2-azobis-2-amidinopropane (AAPH)-induced oxidation of LDL or parinaric acid (PnA) incorporated into isolated human LDL. Interestingly, $(PhSe)_2$ also protected protein moieties from human isolated LDL against Cu^{2+}-induced oxidation. These effects were related to its thiol-peroxidase activity. In experiments with primary cultures of bovine aortic endothelial cells (BAEC), $(PhSe)_2$ protected endothelial cells from the damage promoted by peroxynitrite exposure, in a more effective way than ebselen. The intracellular levels of glutathione (GSH) were almost completely consumed by peroxynitrite and although the compounds did not restore the normal levels, $(PhSe)_2$ *per se* increases significantly GSH in a concentration-dependent manner. This effect may be related with the significant increase in cellular glutathione peroxidase (GPx) activity promoted by this compound. Taken together, the results indicated that $(PhSe)_2$ is relatively safe for rabbits even after a prolonged time of ingestion and suggest a new role for diphenyl diselenide as a potential anti-atherogenic agent.

Key words: Atherosclerosis, diphenyl diselenide, gluthatione peroxidase, hypercholesterolemia, peroxynitrite, rabbits, toxicity.

1 INTRODUÇÃO

1.1 Doenças cardiovasculares e aterosclerose

1.1.1 Aspectos gerais

Atualmente, as doenças cardiovasculares são a principal causa de morbidade e mortalidade nos países desenvolvidos. A extensão deste problema é tal que as doenças cardiovasculares são responsáveis pela perda de mais vidas que todos os tipos de câncer combinados. Apesar de correntemente ser um problema dos países desenvolvidos, a Organização Mundial de Saúde prevê que a prosperidade econômica global possa levar ao alastramento do problema aos países em desenvolvimento, no caso destes adquirirem os hábitos ocidentais.

As doenças cardiovasculares são responsáveis por 38% do total de mortes registados nos Estados Unidos, sendo, na Europa, a causa de morte mais comum em homens com menos de 65 anos e a segunda em mulheres na mesma faixa etária (Hansson, 2006). Apesar do progresso em tratamentos para a hipercolesterolemia e hipertensão, fatores considerados de risco para o desenvolvimento de doenças cardiovasculares, prevê-se que estas continuem a ser a principal causa de morte nos países desenvolvidos, nos próximos 15 anos.

A aterosclerose é a condição patológica que antecede a maior parte dos episódios cardiovasculares, nomeadamente infartos do miocárdio e acidentes vasculares cerebrais, na qual as artérias desenvolvem uma constrição do seu espaço interno e perdem elasticidade (Itabe, 2003). Após várias décadas de estudo concluiu-se que a aterosclerose não se trata de uma simples consequência degenerativa do envelhecimento, mas, de uma condição inflamatória crônica (Ross, 1999). De um modo geral, a aterosclerose caracteriza-se pela acumulação de material lipídico e elementos celulares, especialmente macrófagos e células musculares lisas, na túnica íntima das artérias de médio e grande calibre, levando à formação de placas – placas de ateroma – que gradualmente estreitam o lúmen vascular e dificultam o fluxo sanguíneo. Os vasos sanguíneos mais comumente afetados e clinicamente mais relevantes incluem a artéria aorta e as artérias carótidas, coronárias e cerebrais. O caráter crônico do processo conduz à formação de lesões que podem obstruir completamente o lúmen vascular, diretamente ou mediante complicação trombótica (Lusis, 2000).

1.1.2 Características morfológicas da aterosclerose

A aterosclerose manifesta-se como lesões arteriais, denominadas placas, as quais são caracterizadas em seis tipos principais, refletindo os diferentes estados da doença (Fig. 1). Como evento inicial ao desenvolvimento de uma lesão aterosclerótica, nos locais da parede da artéria mais susceptíveis à ocorrência de lesão, ocorre um espessamento adaptativo da íntima. À medida que os macrófagos acumulam lipídeos, formam-se as lesões tipo II como áreas nodulares de deposição lipídica, também denominadas de “estrias gordurosas”, e representadas pelos macrófagos saturados de lipídeos (“células esponjosas”).

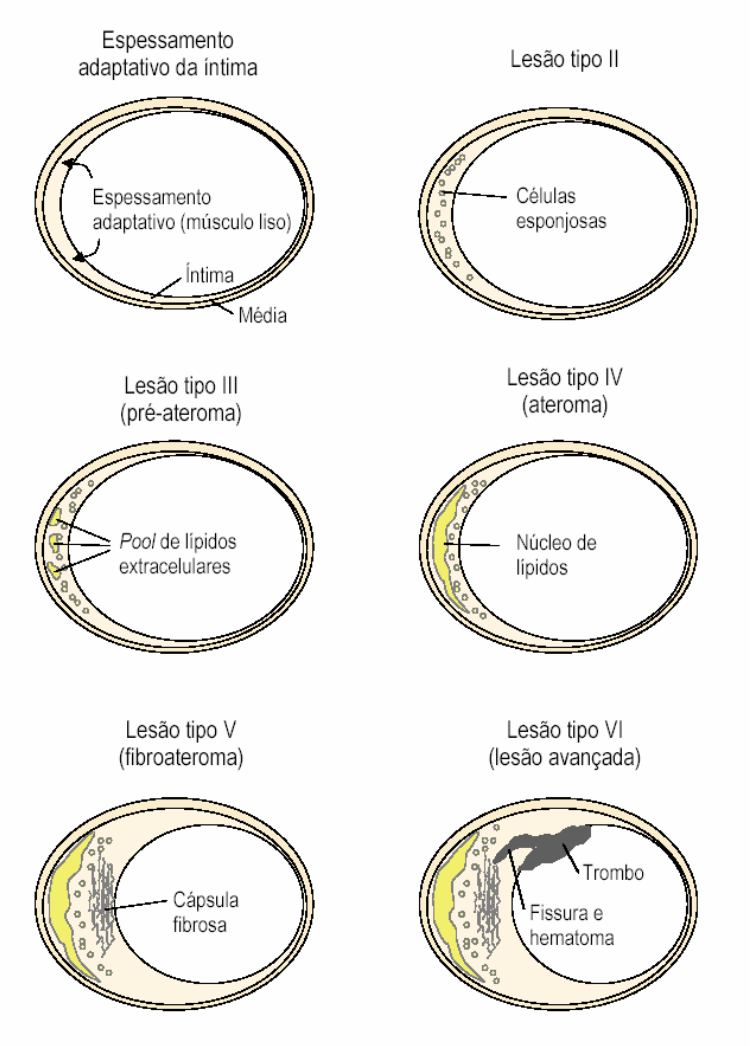

Figura 1. Modificações na estrutura da parede da artéria durante o desenvolvimento da aterosclerose (modificado de Stary et al., 1995).

A continuada formação de células esponjosas e a consequente necrose dos macrófagos resulta em lesões tipo III, lesões compostas por um pequeno pool de lipídeos extracelulares. O desenvolvimento da lesão prossegue com a coalescência dos pequenos aglomerados de lipídeos extracelulares para formar uma área lipídica significativa que representa o centro da lesão aterosclerótica. A lesão tipo IV é definida pela presença de uma camada de tecido consideravelmente fina na separação entre o centro lipídico e o lúmen arterial, enquanto que a lesão tipo V apresenta um espessamento fibroso nesta estrutura (cápsula fibrosa). Uma lesão avançada, lesão tipo VI, exibe uma arquitetura mais complicada, caracterizada por áreas fibrosas calcificadas com ulceração visível. Quando ocorre dano ou ruptura da superfície destas lesões, rapidamente há adesão e agregação de plaquetas e subseqüente geração de trombos. A formação de trombos pode resultar na completa oclusão do vaso, sendo a causa primária de infarto de miocárdio e de acidente vascular cerebral (Strary et al., 1995).

1.1.3 Hipóteses para a Aterogênese

Ao longo de várias décadas, a tentativa de explicação dos complexos eventos celulares e moleculares associados ao desenvolvimento da aterosclerose, resultou na formulação de diversas hipóteses prevendo vias e mecanismos necessários ou suficientes distintos para suportar o desenvolvimento das lesões ateroscleróticas. Os eventos incitadores da doença incluem disfunção endotelial, a retenção das lipoproteínas de baixa densidade (LDL) na matriz endotelial e a modificação oxidativa das LDL. Segundo a hipótese da resposta à injúria, que considera a disfunção endotelial o evento precursor da aterosclerose, esta é vista como uma resposta protetora contra determinado insulto vascular, sendo o desenrolar da doença uma tentativa de remediar a progressão dessa injúria. A hipótese da acumulação de lipídeos, proposta em 1913 por Anitschkow e Chalatow, atribuía a aterosclerose à gradual acumulação de lipídeos na parede arterial (Tiong e Brieger, 2005). Estas hipóteses não são mutuamente exclusivas, apesar de apontarem eventos percursores distintos, todas elas envolvem uma componente significativa de inflamação, uma característica bem conhecida da aterosclerose, e incluem as LDL como elemento central. No entanto, a hipótese da modificação oxidativa propõe um mecanismo molecular numa sequência de eventos, dando particular importância aos eventos oxidativos e às reações redox na gênese da aterosclerose.

1.1.3.1 Hipótese da modificação oxidativa das lipoproteínas de baixa densidade

De acordo com a hipótese da modificação oxidativa das lipoproteínas de baixa densidade (LDL), proposta em 1989 por Steinberg e colaboradores, as LDL oxidadas desempenham um papel fundamental na aterogênese através (I) do recrutamento de monócitos circulantes para a túnica íntima, (II) da inibição da capacidade dos macrófagos residentes abandonarem este espaço, (III) do aumento da taxa de captação destas LDL levando à formação de células esponjosas e (IV) pelo fato de serem citotóxicas, induzirem a perda de integridade endotelial. Esta última propriedade foi proposta para estabelecer a ligação entre a formação das estrias gordurosas e a progressão para lesões mais avançadas (Steinberg et al., 1989).

As LDL são partículas submicroscópicas com massa molecular relativa média de 3000 kDa, com diâmetro entre 22 e 28 nm e densidade entre 1.019 e 1.063 g/ml. A estrutura geral é de uma microemulsão esferoidal formada por uma camada exterior de fosfolipídeospolares, colesterol não esterificado e proteína, com um núcleo central de lipídeos neutros, predominantemente ésteres de colesterol e triacilgliceróis (Fig. 2).

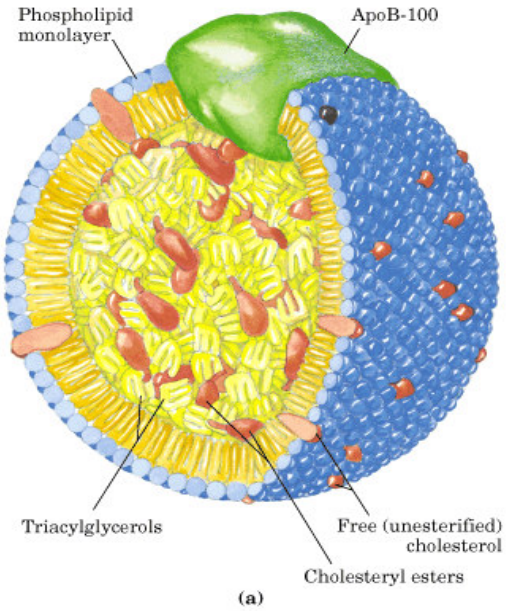

Figura 2. Representação esquemática de uma lipoproteína de baixa densidade (LDL).

Os mecanismos das modificaçoes das lipoproteínas in vivo ainda não estão completamente esclarecidos, todavia, um dos eventos iniciais na oxidação da LDL é a peroxidação dos lipídeos, particularmente, fosfolipídeos e ésteres de colesterol, os quais contêm ácidos graxos poliinsaturados (PUFAs) (Fig. 3). A formação de dienos conjugados ocorre devido à abstração e ao rearranjo molecular do hidrogênio, este dieno conjugado reage muito rapidamente com o oxigênio molecular iniciando uma reação autocatalítica que leva a formação de hidroperóxidos. Os hidroperóxidos lipídicos, por clivagem da ligação carbono-carbono, podem ainda dar origem a fragmentos de cadeia curta, com 3 a 9 carbonos, incluindo aldeídos (malondialdeído e 4-hidroxinonenal) e cetonas, que podem conjugar-se com outros lipídeos ou proteínas (Yamaguchi et al., 2002; Porter, 1984). A presença de peróxidos lipídicos em extratos preparados de aortas ateroscleróticas foi descrita há mais de 50 anos e, desde logo, foi sugerido que a peroxidação lipídica desempenhava um papel ativo na patogênese da aterosclerose (Glavind et al., 1952).

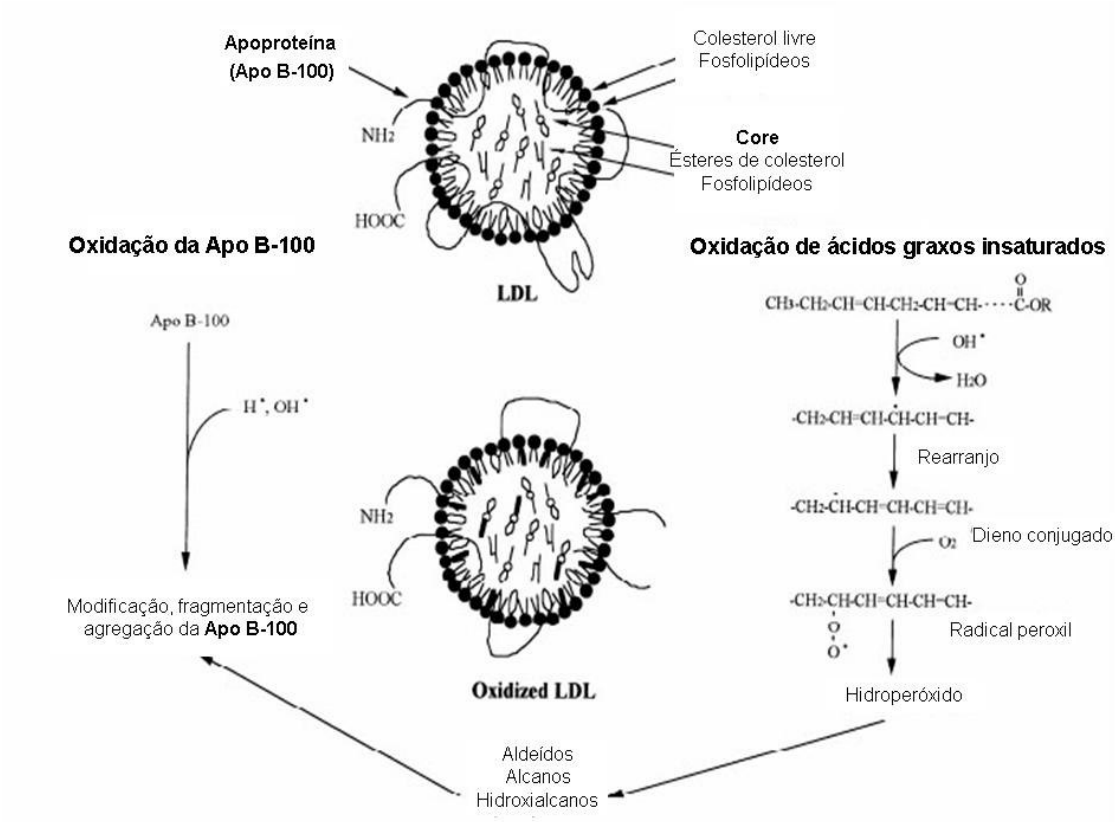

Figura 3. Dano oxidativo à LDL (Modificado de Yamaguchi et al., 2002).

Adicionalmente à oxidação lipídica, a apoproteína B (apo B), maior proteína da LDL fragmenta-se devido à sisão oxidativa (Fig 3). Os produtos formados na lipoperoxidação reagem com resíduos de lisina da apo B, formando bases de Schiff (Steinbrecher et al., 1987; Yamaguchi et al., 2002). Estas modificações resultam quer em alterações conformacionais, pela perda da estrutura secundária, quer em alterações de carga eléctrica, resultando uma proteína com uma carga negativa aumentada. Por sua vez, este aumento na carga global negativa da ApoB resulta no aumento de reconhecimento pelo receptor scavenger dos macrófagos, levando à acumulação descontrolada de LDL por estas células e à consequente formação de células esponjosas, a lesão fisiopatológica primária da aterosclerose.

1.1.3.2 Extensão da oxidação das LDL

Pensa-se que as modificações oxidativas das LDL ocorrem em duas etapas, dando origem a dois tipos funcionais de LDL. Na primeira fase, a oxidação das LDL em pequena extensão resulta na formação de LDL minimamente oxidadas (mmLDL), nesta fase o colesterol ainda é o esterol predominante, a apoB ainda liga aos receptores das LDL e a incubação com macrófagos não resulta na formação de células esponjosas. As mmLDL possuem a capacidade de estimular a produção de proteínas quimiotácticas (por exemplo, MCP-1, proteína quimiotática de monócitos) e de proteínas que promovem a diferenciação e proliferação dos monócitos em macrófagos no espaço subendotelial (M-CSF). Quando as LDL se encontram num estado de oxidação mais avançado, em que a maioria dos ácidos graxos polinsaturados se encontram oxidados numa complexa mistura de produtos e em que a apoB se encontra fragmentada, com conformação alterada e com modificações promovidas pelo aldeídos formados no processo de oxidação, deixam de ser reconhecidas pelos receptores específicos das LDL, passando a ser reconhecidas pelos receptores scavenger (Singh e Jialal, 2006).

1.1.3.3 Mecanismos pelos quais as LDL oxidadas podem ser aterogênicas

Os mecanismos pelos quais as LDL oxidadas podem exercer os seus efeitos aterogênicos são vastos. As LDL oxidadas induzem a formação de células esponjosas derivadas de macrófagos que reconhecem as LDL oxidadas pelos seus receptores scavenger e as captam. Adicionalmente, as LDL oxidadas e seus produtos derivados podem atrair monócitos e macrófagos e induzir a adesão de monócitos ao endotélio, ação que pode potencializar o processo inflamatório

implicado na aterosclerose. Está descrita ainda a capacidade das LDL oxidadas induzirem a migração e proliferação das células musculares lisas, por promoção da expressão de fatores de crescimento, impedirem a migração endotelial necessária para reparação do dano, e ainda a capacidade de causar injúria celular, quer por via necrótica quer por via apoptótica. Por conseguinte, ao promover a disfunção do endotélio, as LDL oxidadas interferem com o relaxamento vascular dependente do endotélio, por indução de perda da bioatividade do óxido nítrico, e, também, pela promoção da atividade procoagulante das células vasculares (Chisolm e Steinberg, 2000).

1.2 Estresse oxidativo e aterosclerose

Recentes estudos têm demonstrado que a utilização alterada do oxigênio e/ou a formação aumentada de espécies reativas de oxigênio (ERO) e de nitrogênio (ERN) contribuem para a progressão da aterogênese e das doenças cardiovasculares. Diversas fontes de ERO e ERN estão presentes na aterosclerose. A geração exagerada destas espécies reativas ultrapassa a capacidade de defesa antioxidante causando a ativação de neutrófilos, peroxidação lipídica, modificação protéica e quebra do DNA. Além disso, ERO/ERN *per se,* bem como seus produtos de oxidação, induzem apoptose do miocárdio (Kaliora et al., 2006).

Modelos animais utilizando coelhos hipercolesterolêmicos demonstraram a primeira evidência indireta do papel da LDL na indução do estresse oxidativo. As aortas destes animais produziram significativamente mais superóxido ($O_2^{\bullet-}$) do que as aortas controles (Ohara et al., 1993; Mügge et al., 1994). Adicionalmente, outros estudos demonstraram que quando células endoteliais, artérias isoladas e outras células sanguíneas foram incubadas com LDL oxidada a formação de $O_2^{\bullet-}$ foi estimulada (Galle et al., 1995; Maeba et al., 1995). Cabe ressaltar ainda que, a LDL é quimicamente vulnerável às ERO, pois possue grande quantidade de ácidos graxos poliinsaturados susceptíveis à oxidação (Kaliora et al., 2006).

1.2.1 Peroxinitrito ($ONOO^-$)

As células vasculares produzem espécies reativas através de enzimas celulares como a NADPH e xantina oxidase, principais fontes de ânions superóxido, e a NO sintase, a qual produz óxido nítrico ($NO^{\bullet}$). Embora o $NO^{\bullet}$ seja uma molécula antiaterogênica devido aos seus efeitos benéficos no endotélio vascular (Cai e Harrison, 2000), ele pode reagir rapidamente com $O_2^{\bullet-}$, levando a formação do

peroxinitrito ($ONOO^-$), um potente agente nitrante e oxidante (Koppenol, 1998). O peroxinitrito pode oxidar e modificar covalentemente uma grande variedade de biomoléculas, incluindo DNA, lipídeos e proteínas, bem como várias biomoléculas de baixo peso melecular (Salgo et al., 1995; Ischiropoulos e Mehdim, 1995; Radi et al., 1991). Estas modificações podem afetar importantes funções celulares e desta forma comprometer a homeostase celular, e subsequentemente gerar processos apoptóticos ou necróticos. O envolvimento do peroxinitrito na aterosclerose tem sido sugerido por sua habilidade em oxidar a LDL e também pela identificação de 3-nitrotirosina em lesões ateroscleróticas (Darley-Usmar et al., 1992; Leeuwenburgh et al. 1997; Rubbo e O'Donnel, 2005; Beckmann et al., 1994).

1.3 Antioxidantes e Aterosclerose

Considerando a "teoria oxidativa da aterosclerose", antioxidantes dietéticos têm atraído considerável atenção como agentes preventivos e terapêuticos. Inúmeras evidências obtidas por estudos in vitro, in vivo, intervensões controladas e estudos em modelos animais demonstram que o consumo de antioxidantes previne a progressão da aterosclerose (Otero et al., 2002; Bleys et al., 2006; Frederiksen et al., 2007)

1.3.1 Selênio (Se)

Selênio é um elemento traço essencial, componente de enzimas com atividades antioxidants, particularmente as isoformas da glutationa peroxidase (Flohe e Gunzler, 1973) e o sistema tireoxina redutase (Engman, 1997). Estes sistemas enzimáticos têm importantes papéis na defesa celular, protegendo contra processos oxidativos possivelmente pela detoxificação de hidroperóxidos lipídicos e ainda apresentam específico envolvimento na defesa contra o peroxinitrito (Sies e Arteel, 2000; Klotz e Sies, 2003). A suplementação com Se demonstrou ser capaz de proteger células endoteliais da injúria oxidativa (Thomas et al., 1993), além disso, há uma correlação inversa entre o status de Se e o risco de doenças cardiovasculares (Huttunen, 1997). Dados da literatura sugerem que o Se pode ser um potente modulador da síntese de prostaciclinas e do metabolismo das lipoproteínas, sugerindo seu potencial anti-aterogênico (Meydani, 1992; Qu et al., 2000, Huang et al., 2002).

1.3.2 Organocalcogênios

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

Conseqüentemente, o risco de contaminação ocupacional por organocalcogênios motiva estudos toxicológicos. Outro aspecto relevante é a tentativa crescente de desenvolvimento de compostos organocalcogênios que possuam atividade biológica e aplicações farmacológicas (Nogueira et al., 2003a).

O conceito de que moléculas contendo selênio podem ser melhores nucleófilos (e, portanto antioxidantes) do que os antioxidantes clássicos têm levado ao desenvolvimento de organocalcogênios sintéticos (Arteel e Sies, 2001). Vários compostos de Se estão sendo apontados como possíveis estratégias terapêuticas para uma grande variedade de doenças, como artrite, hipertensão, dislipidemias, anti-virais, dentre outras (Parnhan e Graf, 1991).

1.3.2.1 Ebselen

O ebselen (2-fenil-1,2-benzilsoselenazol-3(2H)-ona) (Fig. 4) é um composto orgânico de Se cujas propriedades antioxidantes e antiinflamatórias têm merecido destaque no campo da farmacologia. Este composto foi descrito e caracterizado como um mimético da enzima glutationa peroxidase na década de 80 (Muller et al, 1984; Weldel et al.,1984), entretanto, apenas a partir da década de 90, cresceu enormemente o número de trabalhos demosntrando seus efeitos protetores em diferentes tipos celulares e para os mais diversos tipos de injúria.

Dentre os mecanismos moleculares relacionados com os efeitos protetores do ebselen, destacam-se a capacidade de catalisar a redução do peróxido de hidrogênio e de hidroperóxidos orgânicos na presença de glutationa (Muller et al., 1984) e o importante papel na detoxifição peroxinitrito (Masumoto e Sies, 1996).

O Ebselen apresenta baixa toxicidade, porque o átomo de Se de sua molécula não é liberado durante a sua biotransformação e, deste modo, não é metabolizado pelo organismo como outros compostos, em que o Se está mais disponível (Parnhan e Graf, 1990).

Devido às suas propriedades antioxidantes e antiinflamatórias, além da baixa toxicidade, o ebselen tem sido estudado na prevenção e proteção celular contra os mais diversos tipos de injúria em modelos animais, experimentos *in vitro* e inclusive em estudos com humanos (Jozsef and Filep, 2003; Saito et al., 1998; Yamaguchi et al, 1998; Ogawa et al, 1999).

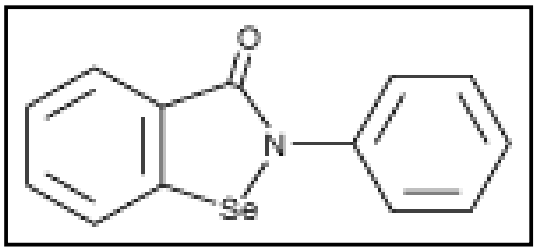

Figura 4- Estrutura química do 2-fenil-1,2-benzilsoselenazol-3(2H)-ona ou Ebselen.

1.3.2.2 Disseleneto de Difenila

O disseleneto de difenila (Fig. 5), assim como o ebselen, é um composto orgânico de selênio que reage eficientemente com hidroperóxidos e peróxidos orgânicos através de reação similar a catalizada pela glutationa peroxidase (GPx). Todavia, o disseleneto de difenila demonstrou ser mais ativo como mimético da glutationa peroxidase (Wilson et al., 1989) e menos tóxico em roedores que o ebselen (Nogueira et al., 2003b; Meotti et al., 2003). Nosso grupo de pesquisa tem demonstrado que o disseleneto de difenila possui importantes propriedades farmacológicas. Um recente estudo demonstrou que o disseleneto de difenila protege plaquetas humanas contra peroxidação lipídica induzida pelo nitroprussiato de sódio, um doador de óxido nítrico, e reativa a atividade da GPx nestas células (Posser et al., 2006) . Adicionamente, o disseleneto de difenila desempenha importante papel protetor em uma variedade de modelos experimentais associados à produção exacerbada de radicais livres (Rossato et al., 2002; Meotti et al, 2004; Ghisleni et al., 2003; Burger et al., 2004; Borges et al., 2005) e demostrou atividades antiinflamatória e antinociceptiva em roedores (Nogueira et al., 2003a; Zasso et al., 2005).

A toxicologia destes compostos é bastante similar. O mecanismo proposto para explicar a toxicidade de compostos de selênio, envolve a oxidação de grupos –SH de moléculas biologicamente ativas (Nogueira et al.,2004). De fato, diversos

trabalhos demonstraram que compostos orgânicos de selênio, inibem um grande número de enzimas sulfidrílicas, incluindo a 5-lipoxigenase (Björnstedt et al., 1996), δ-aminolevulinato desidratase (Nogueira et al., 2003c), esqualeno monooxigenase (Gupta and Porter, 2001) e Na+, K+- ATPase (Borges et al., 2005).

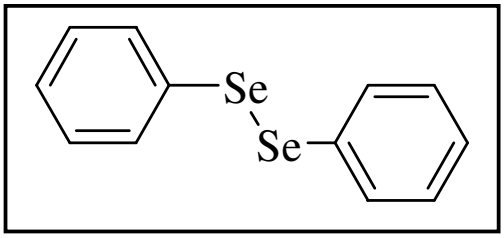

Figura 5- Estrutura química do Disseleneto de Difenila.

2 OBJETIVOS

2.1 Objetivo Geral

Considerando que:

- não existem dados na literatura sobre a avaliação toxicológica do disseleneto de difenila em não roedores;

- o consumo de antioxidante está diretamente relacionado com a diminuição do processo aterosclerótico;

- trabalhos demonstram importante atividade antioxidante do disseleneto de difenila em uma variedade de modelos experimentais associados à produção exacerbada de radicais livres;

- ausência de um completo entendimento sobre os mecanismos moleculares relacionados aos efeitos protetores do disseleneto de difenila contra injúria oxidativa;

- a detoxificação do peroxinitrito está relacionada com o papel protetor dos organocalcogênios.

Este estudo visa:

Avaliar os potenciais efeitos farmacológicos e toxicológicos do disseleneto de difenila em modelos experimemtais *in vivo* e *in vitro*.

2.2 Objetivos Específicos

i) investigar os efeitos toxicológicos *in vivo* relacionados a parâmetros bioquímicos e marcadores do estresse oxidativo no sangue de coelhos durante uma longa suplementação oral com diferentes doses de disseleneto de difenila;

ii) investigar os efeitos toxicológicos *in vivo* relacionados aos marcadores do estresse oxidativo no fígado, rim e cérebro de coelhos após longa suplementação oral com diferentes doses de disseleneto de difenila;

iii) avaliar os efeitos *in vivo* da suplementação oral do disseleneto de difenila nos níveis dos lipídeos plasmáticos e nos marcadores do estresse oxidativo no plasma, fígado e cérebro de coelhos hipercolesterolêmicos;

iv) avaliar o efeito *in vitro* do disseleneto de difenila na proteção contra a oxidação da LDL humana isolada, relacionando o possível mecanismo de ação;

v) investigar o efeito *in vitro* do disseleneto de difenila e do ebselen na morte celular induzida pelo peroxinitrito em cultura primária de células do endotélio de aortas bovinas.

3 ARTIGOS CIENTÍFICOS

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

3.1 Estudos *in vivo*

3.1.1 Efeitos toxicológicos de uma longa suplementação oral de $(PhSe)_2$ em não roedores.

3.1.1.1 Artigo 1

Changes in biochemical parameters in rabbits blood after oral exposure to diphenyl diselenide for long periods

Bem A. F., Portella R.L., Perottoni J., Becker E., Bohrer D., Paixão M. W., Nogueira C. W., Zeni G., Rocha J.B. T*

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Changes in biochemical parameters in rabbits blood after oral exposure to diphenyl diselenide for long periods

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Abstract

The concept that selenium-containing molecules may be better antioxidants than classical antioxidants, has led to the design of synthetic organoselenium compounds. The present study was conducted to evaluate the potential toxicity of long time oral exposure to diphenyl diselenide $(PhSe)_2$ in rabbits. Male adult New Zealand rabbits were divided into four groups, group I served as control; groups II, III and IV received 0.3, 3.0 and 30 ppm of $(PhSe)_2$ pulverized in the chow for 8 months. A number of parameters were examined in blood as indicators of toxicity, including δ-aminolevulinate dehydratase (δ-ALA-D), catalase, glutathione peroxidase (GPx), alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea, creatinine, TBARS, non-protein-SH, ascorbic acid and selenium. The results demonstrated that 6 and 8 months of 30 ppm $(PhSe)_2$ intake caused a significant increase in blood δ-ALA-D activity. Erythrocyte non-protein thiol levels were significantly increased after 2 months of 30 ppm $(PhSe)_2$ intake and then return to control levels after prolonged periods of intake. Ingestion of 3.0 ppm of $(PhSe)_2$ for 8 months significantly increased catalase activity in erythrocytes. Conversely, no alterations in GPx, ALT, AST, TBARS and selenium levels were observed in rabbit serum, conversely, selenium levels in peri-renal adipose tissue were significantly increased after 8 months of 30 ppm $(PhSe)_2$ intake, indicating its great lipophylicity. The present results suggest that diphenyl diselenide was not hepato- or renotoxic for rabbits, but caused some biochemical alterations that can be related to some pro-oxidant activity of the compound (particularly the reduction in Vitamin C).

Keywords: Diphenyl diselenide; Rabbits; Hepatotoxicity; δ-ALA-D; Vitamin C; Selenium

1. Introduction

Selenium is an essential trace element that plays a crucial role in the catalytic center of glutathione peroxidase isoforms [1–5]. Literature data have consistently indicated that low selenium status increases the risk of oxidative damage and cancer [6], whereas selenium supplementation has antioxidant, chemo-protective and anti-cancer activities [7–9]. However, it is well known for a long time that inorganic selenium is highly toxic to several species of mammals [10,11]. The mechanism of selenium toxicity could be related, at least in part, to

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endogenous thiol oxidation and, more recently, investigators have advanced that selenite can be toxic by generating reactive oxygen species during reaction with thiols [12].

The interest in organoselenides chemistry and biochemistry has increased in the last three decades mainly due to the fact that organoselenium compounds having a potential selenol moiety are good antioxidant candidates [13]. Of particular importance, different classes of organoseleniun compounds (including ebselen) exhibit glutathione peroxidase (GSH-px)-like activity and can decompose hydrogen peroxide and alkylperoxides using GSH or other thiol compounds as hydrogen donors [14–19] In fact, Ebselen has anti-inflammatory, antiatherosclerotic, and cytoprotective properties both in vitro and in vivo models [20–30]. Of clinical significance, ebselen has been used with relative success in humans with aneurismal subarachnoid hemorrhage [15] and acute ischemia stroke [16,31].

Recent data from our laboratory have demonstrated that diphenyl diselenide, the simplest of diaryl diselenides, shares some biological activities with ebselen, including in vivo anti-inflammatory, antinociceptive, anti-ulcer, and in vitro neuroprotective and antioxidant activities in different experimental models [18,32–35] and were less toxic to rodents than ebselen [36]. These biological activities can be related, at least in part, to the fact that diphenyl diselenide is also a GPx mimetic compound [14].

Ebselen is a complex molecule and its synthesis is relatively expensive and time-consuming, while the synthesis of diselenides is easy and less expensive than that of Ebselen. In spite of this, exposure of mice to high doses of diselenide depleted-SH and inhibited the sulfhydryl containing enzyme δ-ALA-D from liver, kidney and brain [37,38]. However, data are lacking regarding the toxicity of this compound after long-term exposure by oral route.

In this study, we examined the potential toxicity of long-term exposure to diphenyl diselenide in rabbits by determining the following blood biochemical parameters as possible markers of toxicity: δ-ALA-D, TBARS, non-protein-SH, urea, creatinine, AST, ALT, Vitamin C, catalase, GPx and selenium.

2. Materials and methods

2.1. Materials

Diphenyl diselenide (Fig. 1) was synthesized according to published method [39]. Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed that the compound obtained presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of $(PhSe)_2$ (99.9%) was determined by GC/HPLC. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

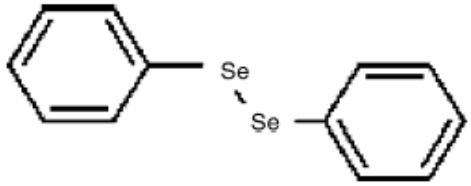

Fig. 1. Structure of diphenyl diselenide.

2.2. Animals and diets

Male (2–3 months old) New Zealand rabbits (1.8–2.2 kg) from our own breeding colony were used. The animals were randomly divided in four experimental groups with six animals per group, and kept one per cage, under a 12:12 h light:dark cycle with free access to food (100 g/day) and water. One group, which served as control, was fed with a standard chow diet (PURINA Type I, Paulínia, Brazil) and three other groups were fed with the same commercial chow supplemented with 0.3; 3.0 and 30 ppm of diphenyl diselenide for 8 months. The control diet was pulverized with ethanol, whereas the supplemented diets were pulverized with diphenyl diselenide dissolved in ethanol. The diets were maintained at 60 °C for 3 h in order to evaporate ethanol. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources of the Federal University Santa Maria. In order to monitor the potential toxicity of diphenyl diselenide as a function of time or exposure, whole blood was collected into heparinized tubes from marginal ear vein of overnight fasted rabbits for determination of δ-ALA-D activity. Blood was collected four times (2, 4, 6 and 8 months after exposure to diphenyl diselenide). Whole blood was centrifuged at 4000 × *g* for 10 min and erythrocytes were used for NPSH and CAT determinations. A parallel blood fraction was collected without anticoagulant and was centrifuged at the same conditions of whole blood to yield serum samples that were used to determine biochemical parameters.

2.3. Hepatic and renal evaluation

Hepatic toxicity was analyzed using serum alanine aminotransferase (ALT) and aspartate aminotransferase

(AST). Renal function was analyzed by determining serum urea and creatinine. The biochemical assays were done using a Johnson & Johnson: Vitros 750 XRC chemistry analyzer.

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

Blood δ-ALA-D activity was assayed according to the method of [40]. The enzyme activity was measured by determining the amount of porphobilinogen formed at 37 °C. The reaction was started by adding substrate (δ-ALA) and incubated for 90 min at 37 °C. The reaction product (porphobilinogen) was determined using modified Ehrlich's reagents and measured at 555 nm.

2.5. Non-protein thiol groups (NPSH) determination

Erythrocyte non-protein thiol groups (NPSH) were determined as described by [59]. Red blood cells pellet (300 μl), obtained after centrifugation of heparinized whole blood, was hemolizated with 100 μl triton 10% solution for 10 min. Then, the protein fraction was precipitated with 200 μl of 10% trichloroacetic acid followed by centrifugation. The colorimetric assay was carried out in phosphate buffer 1 M, pH 7.4. A standard curve using glutathione was constructed in order to calculate the non-protein thiol groups in the tissue samples.

2.6. Determination of TBARS levels

TBARS were determined in plasma by the method of [58], in which malondialdehide (MDA), an end-product of fatty acid peroxidation, reacts with thiobarbituric acid (TBA) to form a colored complex. In brief, samples were incubated at 100 °C for 60 min in acid medium containing 0.45% sodium dodecyl sulfate and 0.6% thiobarbituric acid. After centrifugation the reaction product was determined at 532 nm using 1,1,3,3-tetramethoxypropane as standard and the results were expressed as nmol MDA/ml plasma.

2.7. Ascorbic acid determination

Ascorbic acid determination was performed as described by [38]. Plasmas were precipitated with 1 volume of a cold 10% trichloroacetic acid solution followed by centrifugation. An aliquot of 300 μl of the supernatants were mixed with 2,4-dinitrophenylhydrazine (4.5 mg/ml), $CuSO_4$ (0.075 mg/ml) and trichloroacetic acid 13.3% (final volume 1 ml) and incubated for 3 h at 37 °C. Then 1 ml of H_2SO_4 65% (v/v) was added to the medium. The content of ascorbic acid was calculated using a standard curve (1.5–4.5 μmol/l ascorbic acid freshly prepared in sulfuric acid) and expressed as μmol ascorbic acid/ml of plasma).

2.8. Glutathione peroxidase (GSHPx) assay

GSHPx (EC 1.11.1.9) activity determination was assayed by the method of Pagalia and Valentine (1967). In this method, GSHPx catalyses the oxidation of glutathione in the presence of hydrogen hydroperoxide. Oxidized glutathione is converted to the reduced form in the presence of glutathione reductase and NADPH, while NADPH is oxidized to $NADP^+$. In brief, plasma (10 μl) was added to the assay mixture (total volume = 500 μl) and the reaction started by the addition of H_2O_2 to give a final concentration of 0.4 mM. Conversion of NADPH to $NADP^+$ was monitored continuously at 340 nm for 2 min. GSHPx activity was expressed as μmol of NADPH oxidized per minute per ml of plasma, using an extinction coefficient 6.22×10^6 for NADPH.

2.9. Catalase (CAT) assay

Catalase activity was measured by the method of [57]. Packed erythrocytes were hemolyzed by adding one hundred volumes of distilled water, then, 20 μl of this hemolyzed sample was added to a cuvette and the reaction was started by the addition of 100 μl of freshly prepared 300 mM H_2O_2 in phosphate buffer (50 mM, pH 7.0; total volume of incubation: 1 ml). The rate of H_2O_2 decomposition was measured spectrophotometrically at 240 nm during 120 s. The activity of catalase was expressed as μmol H_2O_2/ml eryth/min.

2.10. Total selenium determination

Absorption measurements were made using a SpectrAA 200 spectrometer (Varian, Australia), equipped with a VGA 77 system for hydride generation equipped with the continuous flow HG system and a GTA 100 for electrothermal atomization. A selenium hollow cathod lamp (Varian, Australia) was employed as radiation source. An air flow oven (Ehret, Germany), a 705 UV Digestor with 500 W Hg lamp (Metrohm, Germany), a Berghof BSB 939-IR subboiling distillation apparatus (Eningen, Germany) and a Digimed pH meter D-20 (São Paulo, Brazil) were used. The water used throughout was

distilled, deionized and further purified by a Milli-Q high purity water device (Millipore, Bedford, USA). Selenite standard solution was prepared by diluting Titrisol (Merck, Darmstadt, Germany) according to the manufacturer's recommendation (1 g/l). Working standard solutions were prepared daily diluting the standards in 6 mol/l HCl for HGAAS and in 0.1 mol/l HNO_3 for GFAAS measurements respectively. Freshly prepared solution of sodium tetrahydroborate (III) ($NaBH_4$) (Merck) was used as a 1% (w/v) solution in 0.1 mol/l NaOH. HCl (36%, 1.19 g/ml) and HNO_3 (63%, 1.14 g/ml) (Merck) were further purified by subboiling distillation. All other reagents used were of analytical-reagent grade. Serum was diluted twice in distilled water and selenium content of this fraction was measured by GFAAS using selenium standards prepared in the methanol/dichloromethane mixture [60].

2.11. Statistical analysis

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

3. Results

3.1. Body weight and food intake

Intake of a basal diet containing different levels of diphenyl diselenide (0, 0.3, 3 or 30 ppm) did not change the body weight gain or the final body weight of the rabbits (data not shown). In this vein, all the animals ate a similar amount of food per day.

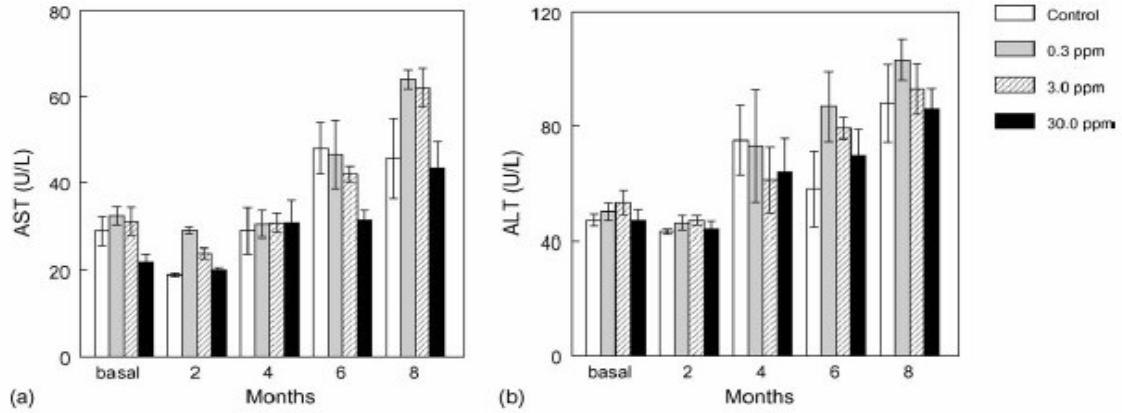

Fig. 2. Rabbit serum ALT and AST activities as a function of time of oral exposure to diphenyl diselenide. Data are expressed as means ± S.E.M. of six animals per group.

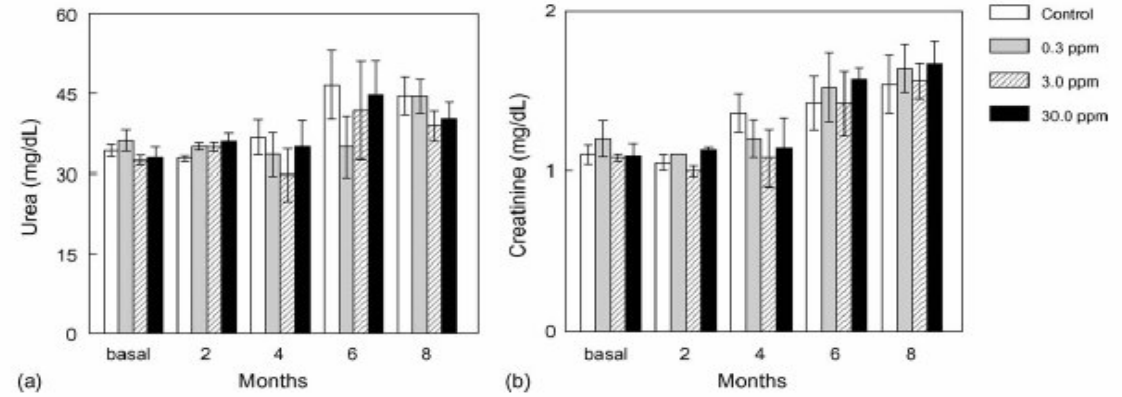

Fig. 3. Rabbit serum urea and creatinine levels as a function of time of oral exposure to diphenyl diselenide. Data are expressed as means ± S.E.M. of six animals per group.

3.2. Transaminase activity (AST and ALT)

Prolonged intake (8 months) of the basal diet supplemented with different levels of diphenyl diselenide (0, 0.3, 3 or 30 ppm) did not modify the activity of ALT and AST, two classical markers of hepatotoxicity in mammals. In accordance with this, the activity of the two enzymes in serum was not modified after 2, 4 or 6 months of exposure to diphenyl diselenide (Fig. 2).

3.3. Creatinine and urea levels

In a similar way to the indicators of hepatotoxicity, the levels of renal toxicity markers (creatinine and urea) were not modified after prolonged intake of diphenyl diselenide (0, 0.3, 3 or 30 ppm). In fact, the levels of creatinine and urea were not modified after 2, 4, 6 or 8 months of exposure to diphenyl diselenide (Fig. 3).

3.4. δ-Aminolevulinic dehydratase (δ-ALA-D) activity

Exposure of rabbits to diphenyl diselenide (0, 0.3, 3 or 30 ppm) for 2 to 8 months was associated with changes in the activity of blood δ-ALA, the increase in enzyme activity was time- and concentration-dependent. In fact, one way ANOVA of blood δ-ALA revealed a significant main effect of $(PhSe)_2$ ($p < 0.05$) and post-hoc comparisons demonstrated a significant increase in blood ALA-D after 6 and 8 month of 30 ppm $(PhSe)_2$ intake ($p < 0.05$; Fig. 4). Ingestion of lower doses of $(PhSe)_2$ (0.3 and 3 ppm) did not change δ-ALA-D activity.

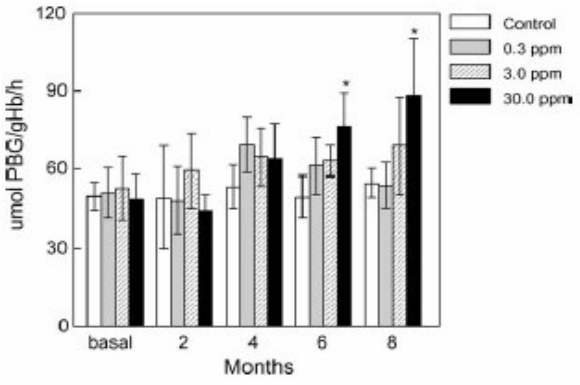

Fig. 4. Rabbit blood δ-ALA-D activity as a function of time of oral exposure to diphenyl diselenide. Data are expressed as means ± S.E.M. of six animals per group. $^*p < 0.05$ as compared to the control group (one-way ANOVA/Duncan).

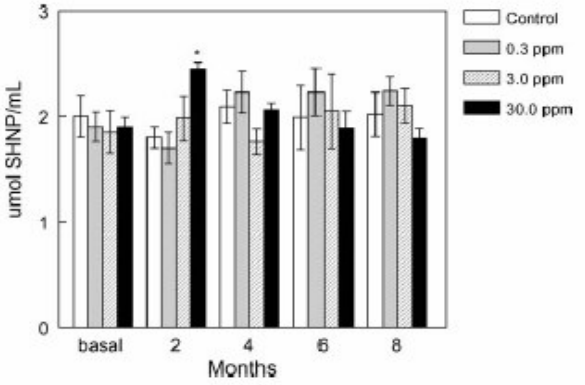

Fig. 5. Rabbit erythrocyte NPSH levels as a function of time of oral exposure to diphenyl diselenide. Data are expressed as means ± S.E.M. of six animals per group. $^*p < 0.05$ as compared to the control group (one-way ANOVA/Duncan).

3.5. Non-protein thiol content—NPSH

Exposure of rabbits to diphenyl diselenide (0, 0.3, 3 or 30 ppm) for 2–8 months was associated with minor and isolated changes in the level of NPSH. In fact, one way ANOVA of erythrocytes NPSH revealed a significant main effect of $(PhSe)_2$ ($p < 0.05$) and post-hoc comparisons demonstrated a significant increase in NPSH only in the group exposed to 30 ppm $(PhSe)_2$ for 2 months ($p < 0.05$; Fig. 5). Ingestion of lower doses of $(PhSe)_2$ (0.3 and 3 ppm) did not change NPSH levels, regardless of the time of exposure.

3.6. TBARS levels

Prolonged intake (8 months) of the basal diet supplemented with different levels of diphenyl diselenide (0, 0.3, 3 or 30 ppm) did not modify TBARS levels, a marker of lipid peroxidation. In accordance with this, the TBARS levels in plasma were not modified after 2, 4 or 6 months of exposure to diphenyl diselenide (Fig. 6).

3.7. Ascorbic acid determination

Exposure of rabbits to diphenyl diselenide (0, 0.3, 3 or 30 ppm) for 8 months was associated with a significant decrease in the levels of ascorbic acid in plasma, which was concentration-dependent. In line with this, one-way ANOVA yielded a significant main effect of $(PhSe)_2$ ($p < 0.05$, Fig. 7) and post-hoc comparisons indicated that ingestion of 30 ppm of $(PhSe)_2$ for 8 months significantly reduced ascorbic acid levels in plasma ($p < 0.05$).

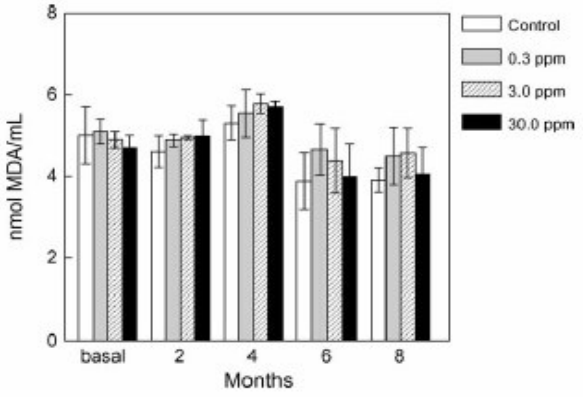

Fig. 6. Rabbit plasma TBARS levels as a function of time of oral exposure to diphenyl diselenide. Data are reported as means ± S.E.M of six animals per group.

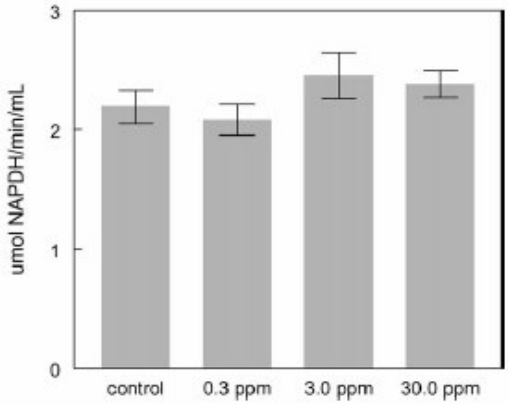

Fig. 8. Effect of 8 months of $(PhSe)_2$ intake on plasma glutathione peroxidase activity in rabbit. Data are reported as mean ± S.E.M of six animals each group.

3.8. Catalase and GPx activities

Exposure of rabbits to diphenyl diselenide (0, 0.3, 3 or 30 ppm) for 8 months was not associated with significant changes in GPx activity. Accordingly, one-way ANOVA of plasma GPx activity determined at 8 months yielded no significant effect of $(PhSe)_2$ ($p > 0.10$) (Fig. 8). In contrast to GPx, one-way ANOVA of erythrocytic catalase activity yielded a significant main effect of $(PhSe)_2$ ($p < 0.05$) (Fig. 9). Post-hoc comparisons indicated that ingestion of 3.0 ppm of $(PhSe)_2$ for 8 months significantly increase catalase activity in erythrocytes ($p < 0.05$). Conversely, no alterations in catalase activity were observed at the other doses tested (Fig. 9).

3.9. Selenium content

Ingestion of $(PhSe)_2$ (0, 0.3, 3 or 30 ppm) for 8 month did not change the levels of selenium in serum (Fig. 10a). In contrast, to serum selenium levels, the deposition of selenium in the peri-renal adipose tissue was significantly increased after ingestion of 30 ppm of $(PhSe)_2$. One-way ANOVA yielded a significant main effect of $(PhSe)_2$ ($p < 0.00001$) (Fig. 10). Post-hoc comparisons indicated that ingestion of 30.0 ppm of $(PhSe)_2$ for 8 months significantly increase selenium levels in peri-renal adipose tissue ($p < 0.00001$). Conversely, no alterations in selenium levels were observed at the other doses tested (Fig. 10).

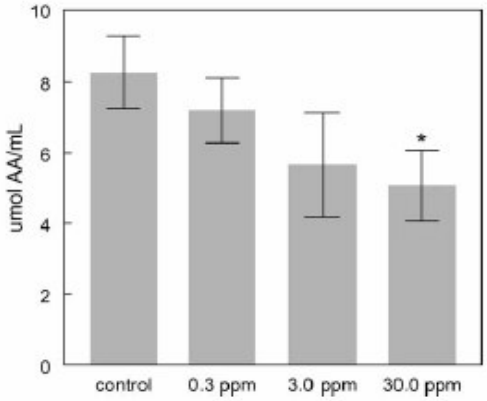

Fig. 7. Effect of 8 months of $(PhSe)_2$ intake on plasma ascorbic acid levels in rabbit. Data are reported as means ± S.E.M of six animals per group. $^*p < 0.05$ as compared to the control group A (one-way ANOVA/Duncan).

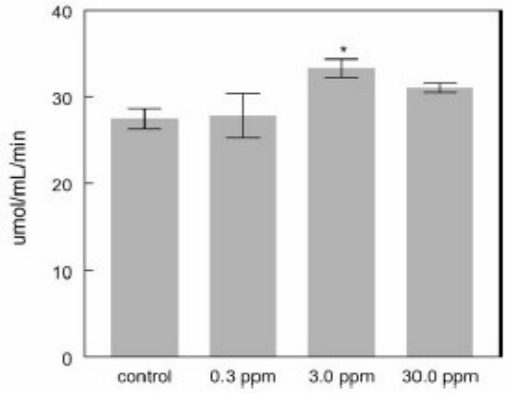

Fig. 9. Effect of 8 months of $(PhSe)_2$ intake on erythrocyte catalase activity in rabbit. Data are reported as means ± S.E.M of six animals per group. $^*p < 0.05$ as compared to control group (one-way ANOVA/Duncan).

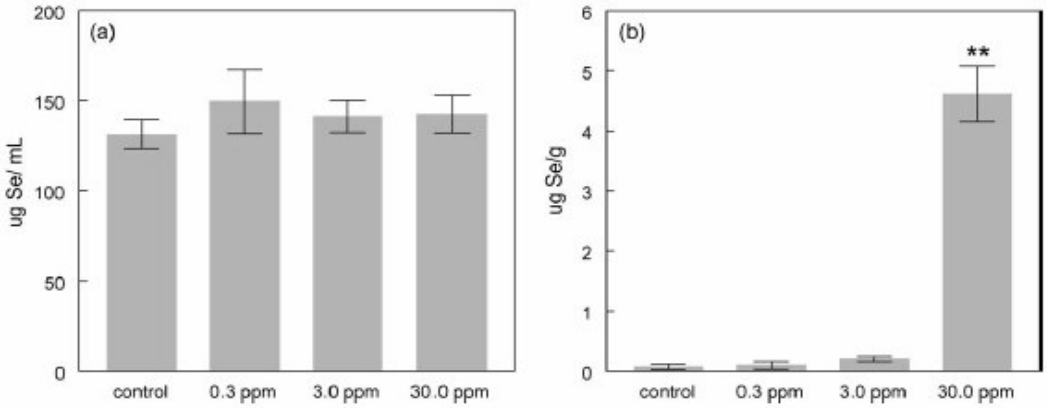

Fig. 10. Effect of 8 months of $(PhSe)_2$ intake on serum selenium levels (a) and in peri-renal adipose tissue in rabbit (b). Data are reported as means ± S.E.M of six animals per group. $^{**}p < 0.0001$ as compared to control group (one-way ANOVA/Duncan).

4. Discussion

The results of the present study suggest that oral exposure to diphenyl diselenide for long periods was relatively without toxic effects to rabbits. In fact, all the biochemical parameters analyzed are little modified by ingestion of diphenyl diselenide. The potential toxicity of diphenyl diselenide to liver that was early observed in mice exposed to high doses of this compound for 10 days [38] was not observed in rabbits. In fact, exposure to 30 ppm of diphenyl diselenide, a dose of selenium that is far high than that required nutritionally, caused no sign of hepatoxicity in rabbits even after a very long period of exposure to the compound.

Of particular importance, recent data from our laboratory have demonstrated that diphenyl diselenide is a safe drug when administered acutely to rats at doses in which it presents anti-inflammatory and antinociceptive activity [32,41]. Species differences in toxicity are often related to differences in the metabolism and disposition of a compound [42,43]. In line with this, previous report demonstrated that the toxicity of diphenyl diselenide depends on the route of administration (i.p. or s.c.) as well as the species (rat or mice) [32].

Diphenyl diselenide has been described as a potential antioxidant agent [34]; however, literature data also indicate that it can have some pro-mutagenic activity [44]. Here, we determine the activity of various biochemical parameters that are indicators of oxidative stress and, from the enzymes evaluated, only catalase was increased in erythrocytes of rabbits exposed to 3 ppm of diphenyl diselenide for 8 months. For non-enzymatic antioxidant parameters, only Vitamin C was consistently reduced by exposure to relatively high doses of diphenyl diselenide. In fact, there was a dose dependent reduction in plasma Vitamin C content, which may indicate a toxic effect of diphenyl diselenide. These results are in sharp contrast to that obtained in mice exposed for 10 days to high doses of diphenyl diselenide, where an increase in the hepatic content of Vitamin C was observed [38]. Taken together, these results clearly indicate that the possible toxic effect of new potential therapeutic drugs should be studied in different species of animal models.

Vitamin C is always considered a marker of oxidative stress and the reduction of its content may indicate an increase in oxidative stress. However, the plasma levels of TBARS, an indirect indicator of lipid peroxidation, were not altered after ingestion of diphenyl diselenide. These results indicate that diphenyl diselenide ingestion is not able to induce lipid peroxidation. In fact, these results are consistent with the in vitro evidence that diphenyl diselenide is an inhibitor of lipid peroxidation [34,18]. So, here, diphenyl diselenide caused a dose dependent reduction in Vitamin C that was not accompanied by an increase in TBARS production.

The vulnerability of the liver to chemical injury is due to its anatomical proximity to the blood supply and digestive tract as to its ability to biotransform and concentrate xenobiotics [45]. In the present work, diphenyl diselenide did not change serum AST and ALT activities. Similarly to liver, diphenyl diselenide did not change the markers of renal function, indeed urea and creatinine levels were not modified by diphenyl diselenide. These data suggest no hepatic and renal toxicity for diselenide in rabbits. These results are different from that obtained early with rodents; where hepatotoxicity were observed after exposure to high dose of diphenyl diselenide [38,37,36].

The antioxidant potential of diphenyl diselenide can be explained in part based on its glutathione peroxidase-

like activity. The reaction catalyzed by organoselenium compound is similar to that catalyzed by glutathione peroxidase [19] and is of particular significance for living cells because it decomposes hydrogen peroxide, an intermediate that can give origin to the extremely reactive and toxic product $OH^{\bullet}$ [46]. In this study, intake of diphenyl diselenide for 8 months did not alter glutathione peroxidase activity. The antioxidant activity of diphenyl diselenide is not only due to its thiol-peroxidase-like activity [19,18] and the absence of an increase in plasma GPx activity after intake of diselenide does not imply that it will have no antioxidant effect. Furthermore, the peroxidase-like activity of organochalcogens is about three orders of magnitude lower than that of GPX_1. Thus, the absence of diphenyl diselenide effect on "total GPx activity" can be related to its small thiol-peroxidase activity when compared to the native enzyme activity. In line with this, there are some studies from our laboratory showing that exposure of mice or rats to ebselen (a compound with similar thiol peroxidase-like activity of diselenide) is not associated with an increase in GPx activity in liver [47,48].

Intake of diphenyl diselenide for 8 months did not increase selenium deposition in rabbit serum. These results indicate that blood did not retain significant amounts of diphenyl diselenide and this is possibly due to organoselenium high liposolubility, which was confirmed by selenium deposition in the peri-renal adipose tissue. Thus, it seems that diphenyl diselenide rapidly re-distribute from blood to lipid-rich tissues.

δ-Aminolevulinate dehydratase is an essential enzyme in most organisms in the biosynthetic pathway of tetrapyrroles such as heme and chlorophyll [49]. Due to its sulfhydrylic nature, δ-ALA-D is inhibited by thiol-oxidizing agents, such as diphenyl diselenide [50,38,51–54]. The enzyme inhibition can lead to the accumulation of δ-aminolevulinic acid, which has some pro-oxidant activities [55,56]. In contrast to our previous studies with mice and rats, here we observed that long-term intake of diphenyl diselenide produced an increase in blood δ-ALA-D. The reasons for the differences are at present unknown but may be related to long-term exposure used here and also to the route, the lower daily dosage of diphenyl diselenide and the species used in the present study.

Taken together, the results indicate diphenyl diselenide is relatively safe for rabbits even after a prolonged time of ingestion. However, it is important to emphasize that at the higher doses tested (3 and 30 ppm) diphenyl diselenide promoted some biochemical alterations (decrease in Vitamin C, increase in catalase and δ-ALA-D) which may be an indicative of some toxic effect of this compound. In spite of this, animals showed no sign of gross toxicity and further studies, using higher doses for short times will be necessary to determine whether diphenyl diselenide is a safe compound to rabbits. However, these results are still too preliminary to indicate that the regular intake of diphenyl diselenide could result in real advantages for mammals.

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3.1.1.2 Artigo 2

Low toxicity of diphenyl diselenide in rabbits: a long-term study

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Low Toxicity of Diphenyl Diselenide in Rabbits: A Long-Term Study

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Abstract: Selenium compounds, like diphenyl diselenide (Ph_2Se_2), possess glutathione peroxidase (GSHPx)-like activities and other antioxidant properties. The aim of this study was to evaluate the effects of a long-term oral supplementation with Ph_2Se_2 on various toxicological parameters in rabbits. Adult New Zealand male rabbits were divided into four groups: Group I served as control; Groups II, III and IV received 0.3, 3.0 and 30 p.p.m. of Ph_2Se_2 pulverized in the chow for 8 months. A number of toxicological parameters were examined in liver, kidney, cerebral cortex and hippocampus, such as δ-aminolaevulinic acid dehydratase (δ-ALA-D), catalase (CAT), GSHPx activities, non-protein thiol (–SH), lipid peroxidation and ascorbic acid levels. The results indicated that supplementation 30 p.p.m. Ph_2Se_2 significantly increased δ-ALA-D activity in liver and in cerebral cortex. Non-protein –SH levels were significantly increased in liver but not in kidney, cerebral cortex and hippocampus of rabbits. Ascorbic acid content was significantly lower in the liver and cerebral cortex after supplementation with 30 p.p.m. Ph_2Se_2. Conversely, no alterations in GSHPx and CAT activities, nor in thiobarbituric acid reactive substances levels were observed in rabbit tissues. These results indicate that oral supplementation with Ph_2Se_2 is relatively secure in rabbits after 8 months of exposure. The findings encourage further experiments on the potential therapeutic effects of such compound.

The balance between pro-oxidants and antioxidants is critical for survival and functioning of aerobic organisms [1]. In line with this, reactive oxygen species (ROS) are implicated in ageing [2] and in a variety of diseases including atherosclerosis [3], respiratory tract disorders [4], neurodegenerative diseases [5], inflammatory bowel disease [6] and cancer [7].

Selenium is an essential trace element that plays a crucial role as an essential component of enzymes with antioxidant properties, particularly in the isoforms of gluthathione peroxidases (GSHPx) [8] and in mammalian thioredoxin system, which has been shown to directly reduce lipid hydroperoxides. It also plays a specific role in peroxynitrite defence [9]. In fact, a recent study has indicated that low selenium status may increase the risk of oxidative damage and cancer [10], while selenium supplementation has chemoprotective and anticancer activities [11].

The concept that selenium-containing molecules may be better nucleophiles (and therefore antioxidants) than 'classical' antioxidants has led to the design of synthetic organoselenium compounds [12], some of them with GSHPx-mimetic activity. The first example of such a compound is ebselen [13], which has been demonstrated to exert anti-inflammatory, anti-atherosclerotic and cytoprotective properties in both *in vitro* and *in vivo* models [14–17]. Furthermore, ebselen possesses very low toxicity because its selenium moiety is not liberated during biotransformation and therefore does not enter the selenium metabolism of the organism [18]. Most importantly, ebselen has been used with relative success in clinical trials for the treatment of patients with aneurismal sub-arachnoid haemorrhage [19] and acute ischaemia stroke [20].

Based on the facts that the pharmacological properties of ebselen are related to its thiol (–SH) peroxidase-like activity and that simple diorganoil chalcogenides are GSHPx-mimetic compounds [21], we are currently investigating the pharmacological and toxicological properties of diphenyl diselenide (Ph_2Se_2). This compound is not toxic to mice and rats in anti-inflammatory and anti-nociceptive doses [22,23]. Furthermore, *in vitro* and *ex vivo* studies have also demonstrated that Ph_2Se_2 is a potential antioxidant compound [24–26]. Additionally, Ph_2Se_2 has a protective role in a variety of experimental models associated with the overproduction of free radicals in brain and liver [27–29] and reported a hepato-protective effect in diabetic rats [30].

While ebselen is a complex molecule and consequently is expensive to synthesize, diselenides are easily synthesized and are structurally simpler than ebselen. Of toxicological significance, exposure of mice to high doses of Ph_2Se_2 can cause hepatotoxicity, –SH depletion in several tissues and inhibition of the sulfhydryl-containing enzyme δ-aminolaevulinic acid dehydratase (δ–ALA-D) [31,32]. However, data about the toxicity of Ph_2Se_2 after long-term exposure via the oral route are rare in the literature [33].

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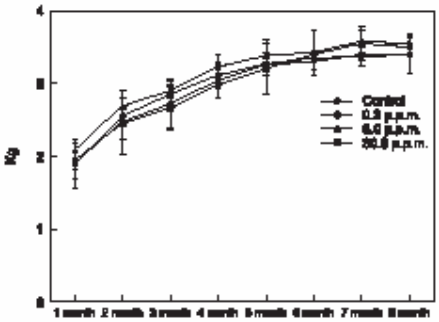

Fig. 1. Weight of animals during the experimental protocol.

The present study was planned to investigate the potential toxic or beneficial effects of Ph_2Se_2 when administrated orally for long periods to rabbits, using a variety of biochemical parameters [thiobarbituric acid reactive substances (TBARS), ascorbic acid, non-protein thiol group (NPSH) levels and δ-ALA-D, catalase (CAT), GSHPx activities] as end-points of organochalcogens toxicity in liver, brain and kidney.

Materials and Methods

Materials. Diphenyl diselenide was synthesized according to previously published methods [34]. Analysis of the ^1H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra showed that the compound obtained presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of Ph_2Se_2 (99.9%) was determined by gas chromatography/high performance liquid chromatography (GC/HPLC). All other chemicals were of analytical grade and were obtained from standard commercial suppliers.

Animals and diets. This experiment complied with the guidelines of the Committee on Care and Use of Experimental Animal Resources of the Federal University of Santa Maria, Brazil. A total of 24 male New Zealand rabbits, weighing 1.8–2.2 kg, were randomly divided in four experimental groups and were kept one per cage, under a 12-hr light:dark cycle with daily access to 100 g of chow and water *ad libitum*. One group, which served as control, was fed with a standard chow diet (Purina Type 1, Paulinia, Brazil) and the three other groups were fed with the same commercial chow supplemented with 0.3, 3.0 and 30 p.p.m. of Ph_2Se_2, for 8 months. The control diet was pulverized with ethanol, whereas the supplemented diets were pulverized with Ph_2Se_2 dissolved in ethanol. The diets were maintained at 60°C for 3 hr in order to evaporate ethanol.

Sample collection. At the end of the eighth month, rabbits were fasted for 12 hr and were killed with an overdose of thiopental (20 mg/kg intravenously). The liver and the kidneys were immediately excised and brain structures (cortex and hippocampus) were carefully separated. Liver, kidney (1 g) and brain structures (0.5 g)

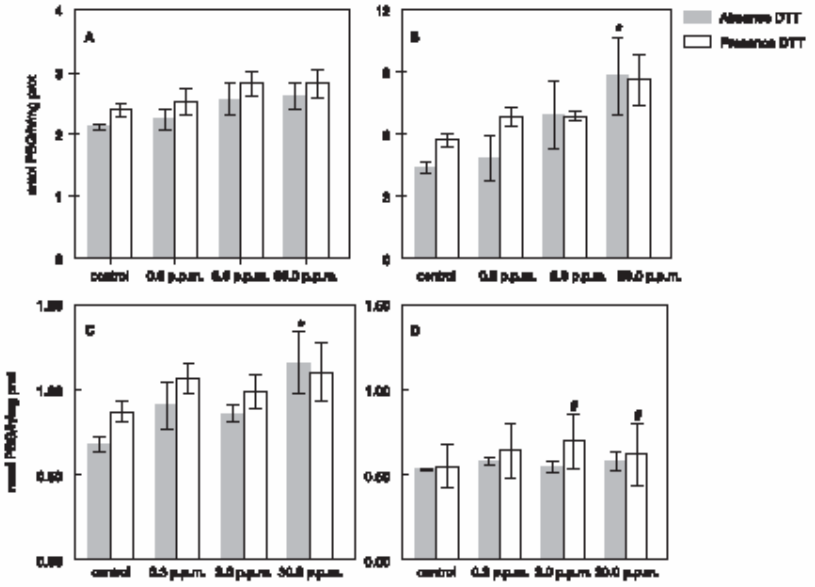

Fig. 2. Effect of Ph_2Se_2 intake for 8 months on renal (A), hepatic (B) cortical (C) and hippocampal (D) δ-aminolaevulinic acid dehydratase (δ-ALA-D) activity in rabbits in absence or presence of dithiotreitol (DTT). Data are expressed as means ± S.E.M. of six animals per group. *denotes $P < 0.05$ as compared to control group in DTT absence, and #denotes $P < 0.05$ as compared to control group in DTT presence (one-way ANOVA/Duncan).

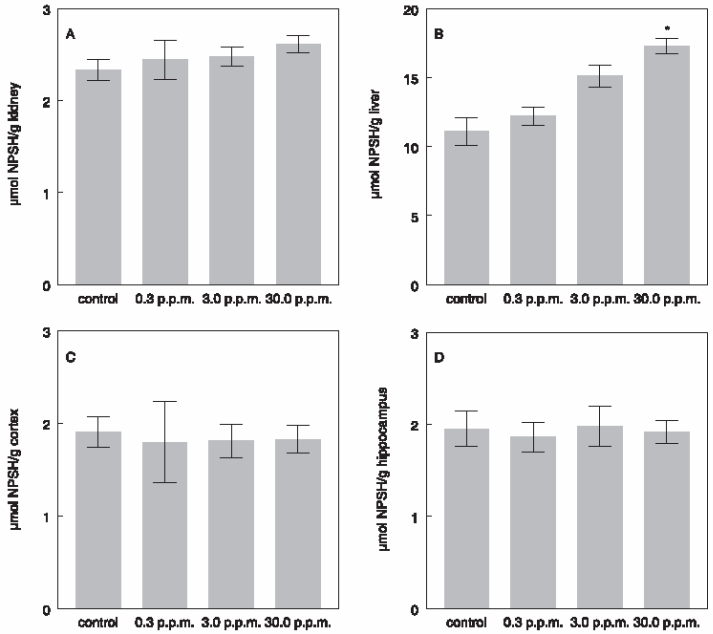

Fig. 3. Effect of Ph_2Se_2 intake for 8 months on renal (A), hepatic (B), brain cortical (C) and hippocampal (D) non-protein thiol groups (NPSH) levels in rabbits. Data are reported as mean ± S.E.M. of six animals per group. *denotes $P < 0.05$ as compared to control group (one-way ANOVA/Duncan).

were homogenized in 10 ml Tris-HCl buffer (10 mM pH 7.4). The homogenates were centrifuged at 3000 ×g for 10 min. to yield a low-speed supernatant, which was used to determine the biochemical parameters. Protein concentration was assayed according to Lowry et al. [35] using bovine albumin as standard.

δ-ALA-D activity. Hepatic, renal and cerebral δ-ALA-D activity was assayed according to the method of Sassa [36], by measuring the rate of product porphobilinogen formation, except that 45 mmol/l sodium phosphate buffer and 2.2 mmol/l δ-ALA-D were used. An aliquot of 100 µl of supernatant was incubated for 1 hr (liver and kidney) and 3 hr (brain structures) at 37°C. The reaction product was determined using modified Ehrlich's reagent at 555 nm. Simultaneously, a set of tubes were assayed in the presence of 1.4 mmol/l dithiothreitol to obtain the reactivation index. This index indicates the extent of the reactivation of δ-ALA-D activity and can indicate the oxidation state of its –SH groups.

NPSH determination. Non-protein thiol groups were determined as described by Ellman [37]. NPSH groups were determined in the fraction obtained after dilution of supernatants with 1 volume of 10% trichloroacetic acid followed by centrifugation. An aliquot of supernatant was added to phosphate buffer (final concentration of 800 mmol/l, pH 7.4) and 500 µmol/l DTNB (5,5′-dithio-*bis*-2-nitrobenzoic acid). Colour development resulting from the reaction between DTNB and thiols reached a maximum in 5 min. and was stable for more than 30 min. Absorbance was read at 412 nm after 10 min. A standard curve using glutathione was constructed in order to calculate the NPSH groups in the tissue samples.

Ascorbic acid determination. Ascorbic acid determination was performed as described by Jacques-Silva et al. [32]. Supernatants were precipitated with 1 volume of a cold 10% trichloroacetic acid solution followed by centrifugation. An aliquot of 300 µl of supernatants was mixed with 2,4-dinitrophenylhydrazine (4.5 mg/ml), $CuSO_4$ (0.075 mg/ml) and trichloroacetic acid 1.5% in a final volume of 1 ml, and incubated for 3 hr at 37°C. Then, 1 ml of H_2SO_4 65% (v/v) was added to the medium. The content of ascorbic acid was calculated using a standard curve (1.5–4.5 µmol/l ascorbic acid freshly prepared in sulfuric acid) and expressed as µmol ascorbic acid/g wet tissue.

Determination of TBARS levels. Thiobarbituric acid reactive substances were determined in the supernatant by the method of Ohkawa et al. [38], in which malondialdehide, an end-product of fatty acid peroxidation, reacts with thiobarbituric acid to form a coloured complex. In brief, samples were incubated at 100°C for 60 min. in a medium containing 0.45% sodium dodecyl sulfate, 100 mM hydrochloric acid, 1.4 M acetic acid, pH 3.4, and 0.6% thiobarbituric acid. After centrifugation the reaction product was determined at 532 nm using 1,1,3,3-tetramethoxypropane as standard.

GSHPx assay. GSHPx was determined by the method of Paglia and Valentine [39]. Tissue supernatants (200–400 µg protein) were added to the assay mixture consisting of (in mmol/l) 1 sodium azide, 1 GSH, 50 potassium phosphate (pH 7.0), and 0.1 unit of glutathione reductase. Reaction was started by the addition of hydrogen peroxide (H_2O_2) to give a final concentration of 0.4 mmol/l. Conversion of NADPH to nicotinamide adenine dinucleotide phosphate ($NADP^+$) was monitored continuously at 340 nm for 2 min.

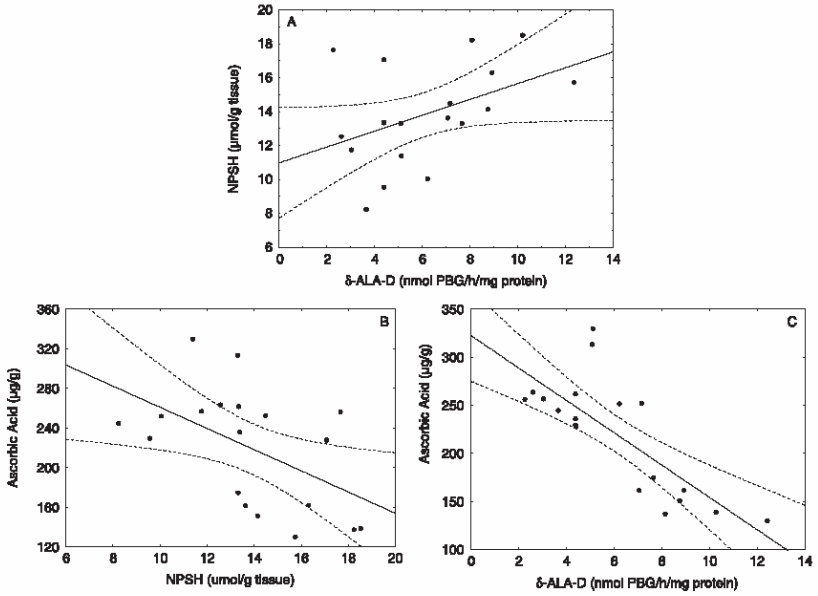

Fig. 4. Significant correlation between (A) non-protein thiol group (NPSH) levels and δ-aminolaevulinic acid dehydratase (δ-ALA-D) activity (r = 0.4410, P = 0.039), (B) ascorbic acid and NPSH levels (r = –0.5181, P = 0.023) and (C) ascorbic acid levels and δ-ALA-D activity (r = –0.77, P = 0.000) in liver of rabbits after Ph_2Se_2 intake for 8 months.

GSHPx activity was expressed as nmol of NADPH oxidized per minute per milligram of protein, using an extinction coefficient 6.22×10^6 M/cm for NADPH.

CAT assay. Catalase activity was measured by the method of Aebi [40]. Supernatants (200–500 µg protein) was added to a cuvette containing phosphate buffer (50 mM, pH 7.0), and the reaction was started by the addition of freshly prepared H_2O_2 to a final concentration of 3 mmol/l. The rate of H_2O_2 decomposition was measured spectrophotometrically at 240 nm during 2 min. The activity of CAT was expressed as µmol H_2O_2 decomposed per minute per milligram of protein, using an extinction coefficient 43.6 M/cm for H_2O_2.

Statistical analysis. Data are expressed as means ± S.E.M. Statistical analysis was performed using a one-way ANOVA, followed by Duncan's multiple range test when appropriate. The Pearson's correlations

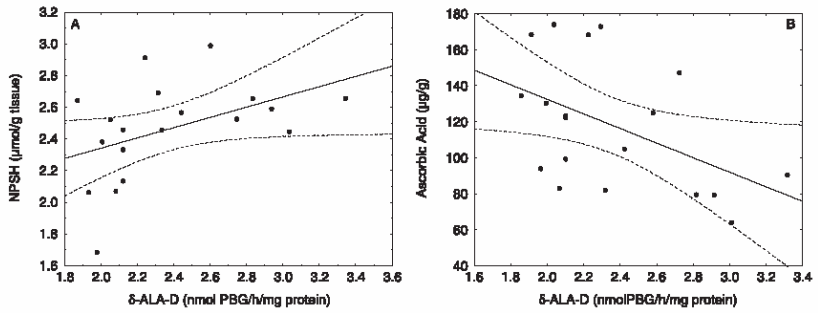

Fig. 5. Significant correlation between (A) non-protein thiol group (NPSH) levels and δ-aminolaevulinic acid dehydratase (δ-ALA-D) activity (r = 0.4433, P = 0.047) and (B) ascorbic acid levels and δ-ALA-D activity (r = –0.4787, P = 0.038) in kidney of rabbits after Ph_2Se_2 intake for 8 months.

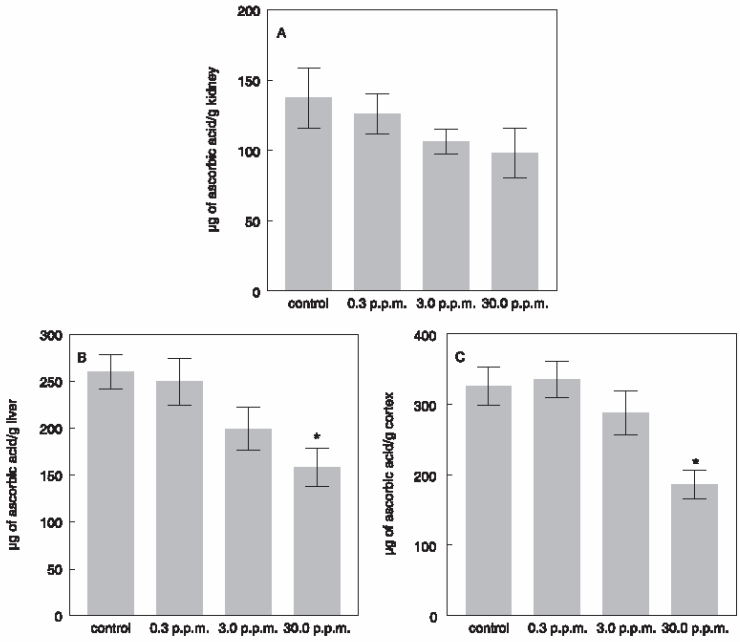

Fig. 6. Effect of Ph_2Se_2 intake for 8 months on renal (A), hepatic (B) and brain cortical (C) ascorbic acid levels in rabbits. Data are reported as mean ± S.E.M. for six animals per group. *denotes $P < 0.05$ as compared to control group (one-way ANOVA/Duncan).

(r, P) in each tissue were calculated for associations among all dependent variables. Values of $P < 0.05$ were considered significant.

Results

Intake of a basal diet containing different levels of Ph_2Se_2 (0, 0.3, 3 or 30 p.p.m.) did not change the body weight gain or the final body weight of the rabbits (fig. 1).

δ-ALA-D activity.

One-way ANOVA of hepatic and brain cortical δ-ALA-D revealed a significant main effect of Ph_2Se_2 ($P < 0.05$). *Post hoc* comparisons demonstrated that long-term intake of 30 p.p.m. Ph_2Se_2 significantly increased ALA-D activity in liver ($P < 0.05$; fig. 2B) and in cerebral cortex (fig. 2C). However, Ph_2Se_2 ingestion did not change renal and hippocampal δ-ALA-D activity (fig. 2A and D). Additionally, the intake of 3.0 and 30 p.p.m. of Ph_2Se_2 for 8 months caused a significant increase in hippocampal δ-ALA-D activity when dithiothreitol was presented in the reaction medium (fig. 2D).

Non-protein –SH.

One-way ANOVA of hepatic NPSH revealed a significant main effect of Ph_2Se_2 ($P < 0.05$). *Post hoc* comparisons demonstrated that long-term intake of 30 p.p.m. Ph_2Se_2 significantly increased NPSH in liver ($P < 0.05$; fig. 3B). In contrast, the ingestion of Ph_2Se_2 for 8 months did not change NPSH levels in kidneys, cerebral cortex and hippocampus from rabbits (fig. 3A, C and D). A positive correlation between NPSH and δ-ALA-D activity in liver and kidney was found to be significant at the 0.05 level (two-tailed Pearson's correlation) when all groups were analysed together (figs 4A and 5A). Contrasting, a negative correlation was observed between NPSH and ascorbic acid levels in liver of rabbits exposed to Ph_2Se_2 (fig. 4B).

Ascorbic acid levels.

Exposure of rabbits to Ph_2Se_2 (0, 0.3, 3 or 30 p.p.m.) for 8 months was associated with a significant decrease in the levels of ascorbic acid in hepatic and brain cortical tissues. In line with this, one-way ANOVA of brain cortical and hepatic ascorbic acid levels yielded a significant main effect of Ph_2Se_2 ($P < 0.05$). *Post hoc* comparisons indicated that ingestion of 30 p.p.m. of Ph_2Se_2 significantly reduced ascorbic acid levels in liver ($P < 0.05$) and cerebral cortex ($P < 0.05$; fig. 6B and C). There was no alteration in ascorbic acid levels in kidney after Ph_2Se_2 exposure (fig. 6A). Interestingly, we found a negative correlation between hepatic and renal ascorbic acid levels and ALA-D activity (figs 4C and 5B).

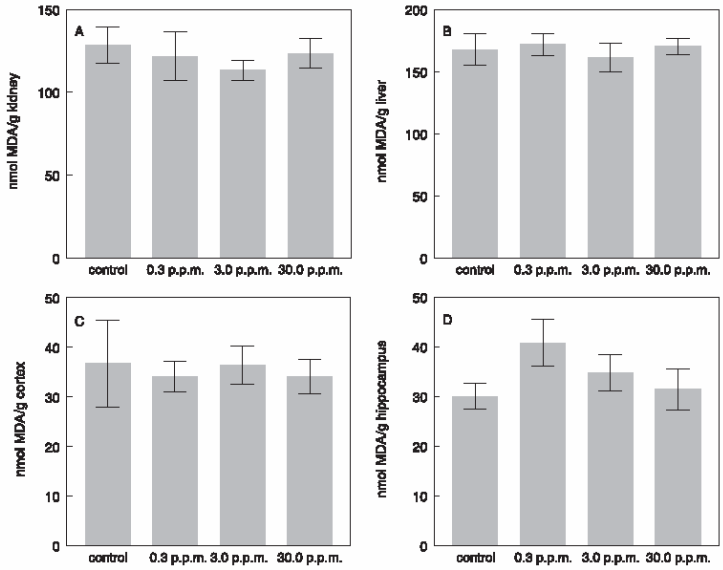

Fig. 7. Effect of Ph_2Se_2 intake for 8 months on renal (A), hepatic (B), cortical (C) and hippocampal (D) lipid peroxidation levels in rabbits. Data are reported as mean ± S.E.M. for six animals per group.

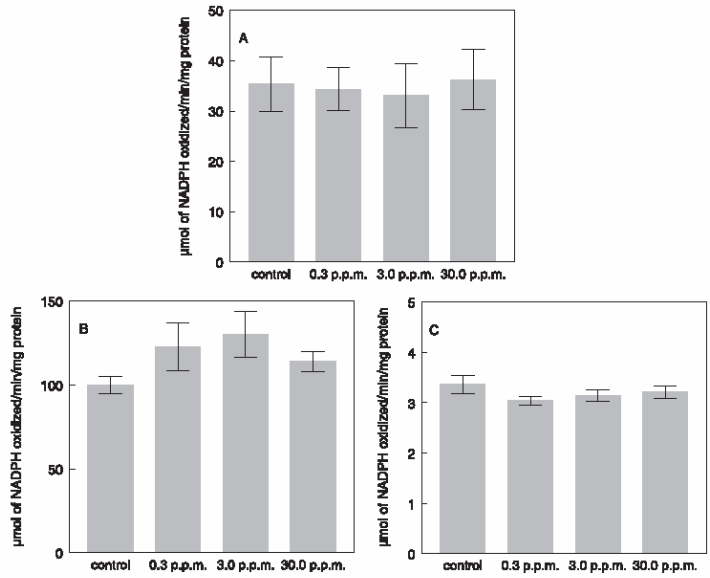

Fig. 8. Effect of Ph_2Se_2 intake for 8 months on renal (A), hepatic (B) and cortical (C) glutathione peroxidase (GSHPx) activity in rabbits. Data are reported as mean ± S.E.M. of six animals per group.

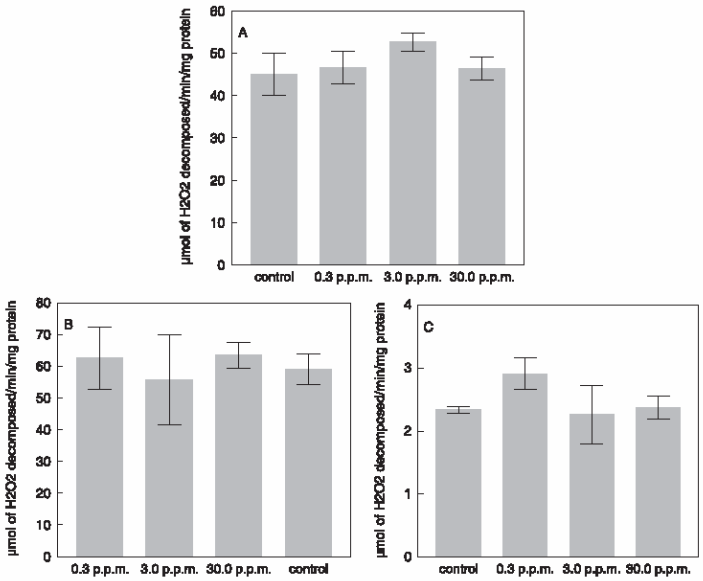

Fig. 9. Effect of Ph_2Se_2 intake for 8 months on renal (A), hepatic (B) and cortical (C) catalase (CAT) activity in rabbits. Data are reported as mean ± S.E.M. of six animals per group.

TBARS levels.

Prolonged intake (8 months) of the basal diet supplemented with different levels of Ph_2Se_2 (0, 0.3, 3 or 30 p.p.m.) did not modify TBARS levels, a marker of lipid peroxidation. In accordance with this, no statistically significant differences were found in TBARS levels in liver, kidney, cerebral cortex and hippocampus of rabbits fed diets containing different levels of Ph_2Se_2 when compared to control rabbits (fig. 7).

Catalase and GSHPx activities.

Exposure of rabbits to Ph_2Se_2 (0, 0.3, 3 or 30 p.p.m.) for 8 months was not associated with significant changes in GHSPx and CAT activities. No statistically significant differences were found in GSHPx and CAT activities in liver, kidney and cerebral cortex of rabbits fed diets containing different levels of Ph_2Se_2, when compared to rabbits fed the control diet (figs 8 and 9).

Discussion

Diphenyl diselenide is a simple diorganoil–selenium compound, which has been demonstrated to exhibit neuroprotective and antioxidant activities *in vitro* [24–26,41] and *in vivo* [27–30,42]. However, this compound can be toxic to rodents after exposure to very high doses [31,32]. The mechanism involved in the toxicity of this compound is related to its interaction with –SH groups of sulfhydryl-containing enzymes, such as δ-ALA-D. In fact, δ-ALA-D is inhibited after both *in vitro* or *in vivo* exposure to this compound [31,32,43], and in mice there was a decrease in non-protein –SH contents in liver after chronic exposure to Ph_2Se_2 [31]. In contrast to these studies, here we observed that intake of 30 p.p.m. of Ph_2Se_2 for 8 months produced an increase in hepatic and cerebral δ-ALA-D activity and in hepatic NPSH levels. In the same way, Ph_2Se_2 produced an increase in blood δ-ALA-D activity after 6 and 8 months of exposure [33]. The increase in liver δ-ALA-D activity may be related to the increase in NPSH found in this tissue. In fact, δ-ALA-D activity has been shown to be modulated by the –SH status, and this phenomenon appears to be related to the presence of sulfhydryl groups located at the active centre of the enzyme [44]. In line with this, there was a positive correlation between δ-ALA-D and NPSH. In kidney, the treatment with Ph_2Se_2 did not change NPSH and δ-ALA-D activity, nevertheless, we found a positive correlation between δ-ALA-D and NPSH in this tissue. In brain, the effect of Ph_2Se_2 was heterogeneous and caused a significant increase in cortical δ-ALA-D, whereas in hippocampus this effect was only observed in the presence of dithiotreitol. In contrast to liver, no increase in NPSH was observed in brain regions, indicating that the increase in enzyme activity was not associated with changes in NPSH levels.

Some observations have suggested that ascorbic acid may play an important role in selenium detoxification [45]. Accordingly, the results of the present study show a marked inhibitory effect of long-term intake of 30 p.p.m. of Ph_2Se_2 in the hepatic and cerebral ascorbic acid levels, whereas in

the kidney, the ascorbic acid content was not modified. These results contrast with those reported by Jaques-Silva et al. [32], who reported an increase in the hepatic ascorbic acid levels in mice exposed subcutaneously for 10 days to high doses of Ph_2Se_2. On the other hand, Ph_2Se_2 did not modify parameters related to oxidative stress, including TBARS and the activity of antioxidant enzymes (GSHPx and CAT). Therefore, the present study suggests that the toxicity of Ph_2Se_2 depends on the route of administration as well as on the species of animal considered [22,46].

In conclusion, the results presented in this study revealed that Ph_2Se_2, administered orally, produced minor toxicological effects in rabbits exposed for a long time. Indeed, only the highest Ph_2Se_2 dose (30 p.p.m.) was able to induce minor changes in biochemical markers of oxidative damage, pointing to a relative safety of such compound when administered during 8 months in rabbits. The toxic potential of Ph_2Se_2 (30 p.p.m.) was evidenced by a decrease in the ascorbic acid levels. Additionally, this compound may be useful as an anti-inflammatory, antinociceptive and neuroprotective agent in mice, in a very similar dose that we used previously [22,27]. Taken together, we suggest that Ph_2Se_2 is a promising compound for future pharmacological studies.

Acknowledgements

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3.1.2 Efeito da suplementação oral de $(PhSe)_2$ em coelhos hipercolesterolêmicos.

3.1.2.1 Artigo 3

Diphenyl diselenide decreases serum levels of total cholesterol and tissue oxidative stress in cholesterol-fed rabbits

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Diphenyl diselenide decreases serum levels of total cholesterol and tissue oxidative stress in cholesterol-fed rabbits

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Abstract

Hypercholesterolemia is one of the major risk factors for coronary artery disease. In addition, cumulative evidences indicate that oxidative stress is involved in all stages of atherosclerosis. Diphenyl diselenide ($(PhSe)_2$ or DD) is a synthetic organoselenium compound that has been shown to have *in vitro* and *in vivo* antioxidant properties. Here we verified whether *in vivo* $(PhSe)_2$ could reduce the hypercholesterolemia and diminish the tissue oxidative stress in cholesterol-fed rabbits. Twenty-four New Zealand white male rabbits were assigned randomly into four groups. The control group was fed a regular chow; the cholesterol group was fed an 1% cholesterol-enriched diet; the DD group was fed a regular diet supplemented with 10 ppm $(PhSe)_2$; and the Chol/DD group was fed the same cholesterol-rich diet which was supplemented with 10 ppm $(PhSe)_2$. Blood samples were collected before (baseline) and after 25 and 45 days of treatment. At the end of the experimental period, the rabbits were killed and the liver and brain were removed and homogenized for laboratory analysis. The results showed that the serum levels of total cholesterol were markedly increased in cholesterol-fed rabbits. Consumption of $(PhSe)_2$ decreased approx 2-fold the serum cholesterol levels in Chol/DD rabbits ($p<0.05$). The intake of $(PhSe)_2$ by hypercholesterolemic rabbits also diminished significantly the serum and hepatic levels of TBARS as well as the production of free radical species in blood and brain ($p<0.05$). In addition, $(PhSe)_2$ supplementation increased hepatic and cerebral δ-aminolevulinic dehydratase activity and hepatic NPSH levels despite of hypercholesterolemia ($p<0.05$). In summary, the results showed that $(PhSe)_2$ might inhibit the hypercholesterolemia and the oxidative stress in cholesterol-fed rabbits.

1 Introduction

Hyperlipidemia, particularly the high levels of serum cholesterol, is one of the major risk factors for atherosclerosis. Free radical-induced lipid peroxidation has been also implicated in the pathogenesis of atherosclerosis, and reactive oxygen species (ROS) are known to be the initiators of lipid peroxidation (Penn and Chisolm, 1994). Previous studies have shown that hyperlipidemia increases the plasma levels of oxygen free radicals, which react with lipids, proteins and DNA to produce oxidized compounds, such as malondialdehyde (MDA), carbonyl compounds and 8-hydroxy-2V-deoxyguanosine (8-OhdG), respectively (Prasad and Kalra, 1993).

Feeding a cholesterol-supplemented diet to rabbits produces severe hypercholesterolemia, causes increased lipid peroxidation, exposing therefore the animals to oxidative stress (Sulyok et al., 1984). The addition of antioxidants to a diet supplemented with cholesterol significantly decreased the plasma lipid peroxidation products as well as the severity of atherosclerotic lesions in rabbits (Wojcicki, et al., 1991, Negis et al, 2006; Jenner et al, 2007).

Selenium (Se) is essential for the expression of several peroxidases and redox enzyme system, which protect cells from oxidative stress (Flohe et al., 1973; Engman et al., 1997). The antioxidant properties of selenium certainly contribute to preserve health conditions and its deficiency has been linked to an increase in the incidence of oxidative stress, cardiovascular disease, immune dysfunctions, cancer, seizures and diabetes (Huang et al., 2002; Navarro-Alarcón et al., 2000; Rayman, 2000; El-Bayoumy, 2001). In addition, Dhingra and Bansal (2006) showed recently that selenium supplementation in the diet decreased the serum levels of total cholesterol and apolipoprotein B-100 and the HMG-CoA reductase mRNA expression in cholesterol-fed rats.

Diphenyl diselenide $(PhSe)_2$ is a synthetic organoselenium compound that has thiol-peroxidase activity and other antioxidant properties (Nogueira et al., 2004). It has been suggested, therefore, that $(PhSe)_2$ can be considered a potent pharmacological agent. Recently, we showed that $(PhSe)_2$ causes a significant reduction in the levels of blood glucose and glycated proteins in diabetic rats (Barbosa et al., 2006). Furthermore, the antioxidant ability of $(PhSe)_2$ was already demonstrated in several in vitro systems containing different tissues, including brain, liver and platelets (Rossato et al., 2002; Meotii et al., 2004; Posser et al. 2006). Of

particular importance is the fact that $(PhSe)_2$ has low toxicity in rabbits after orally long term exposure (Bem et al., 2006; 2007) or after acute administration of supra pharmacological doses to rodents (Nogueira et al., 2003). However, to our knowledge the potential hipocholesterolemic effect of $(PhSe)_2$ has not been yet described.

Therefore, the aim of this study was to examine the effects of $(PhSe)_2$ consumption on the serum lipid and on plasma and tissue markers of oxidative stress in cholesterol-fed rabbits.

2 Materials and methods

2.1 Materials

$(PhSe)_2$ was synthesized according to published methods (Paulmier, 1986). Analysis of the ^1H NMR and ^{13}C NMR spectra showed that the compound obtained presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of $(PhSe)_2$ (99.9%) was determined by GC/HPLC. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.2 Animals and diets

This study complied with the guidelines of the Committee on Care and Use of Experimental Animal Resources of the Federal University of Santa Maria – RS, Brazil. After 1 week of adaptation, male New Zealand white rabbits weighing 1.8–2.2 kg were housed in individual cages, under a 12:12 h light:dark cycle with daily access to 100 g of chow and water ad libitum. The rabbits were randomly divided into four experimental groups (n = 6 for each) and were fed: i) a standard diet chow (PURINA® Type I, Paulínia-SP, Brazil) (Control group); ii) 1% cholesterol-rich diet (Chol group); iii) standard chow plus 10 ppm $(PhSe)_2$ (DD group) and; iv) 1% cholesterol enriched diet plus 10 ppm $(PhSe)_2$ (Chol/DD group). The standard diet was pulverized with diethylether, whereas the supplemented diets were pulverized with cholesterol and/or $(PhSe)_2$ dissolved in ether. The standard and supplemented diets were kept at 60º C for 3 h in order to evaporate ether. The dose of $(PhSe)_2$ chosen for treating animals was based on previous study, which demonstrated that $(PhSe)_2$ did not cause overt signals of toxicity (Bem et al., 2006; 2007). Blood samples were collected from the ear vein of 12 h fasted rabbits before and after 25 days of treatment for biochemical

analyses. After 45 days of dietary treatment, the rabbits were food-deprived for one night and blood was collected by cardiac puncture into heparinized tubes or tubes without additives and then the animals were killed with an intravenous overdose of thiopental. Liver and brain were immediately excised, rinsed and homogenized in 10 vol of 10 mM Tris/HCl buffer, pH 7.4. The homogenates were centrifuged at 3000 x g for 10 min and the supernatant was used to biochemical analyses. Protein concentration in homogenates was assayed according to Lowry et al. (1951) using bovine albumin as standard.

Heparinized blood was used for determination of δ-aminolevulinic dehydratase (δ-ALA-D) activity and reactive species. Blood sample collected without anticoagulant was centrifuged at the same conditions of heparinized blood to yield serum, which was used to the measurement of other biochemical parameters.

2.3 Serum lipids

Serum total cholesterol and triglycerides were determined by enzymatic methods based on the oxidase/peroxidase system, using commercial kit reagents (Labtest Diagnostica®, Lagoa Santa-MG, Brazil).

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

δ-ALA-D activity was assayed according to the method of Sassa (1982). The enzyme activity was determined by measuring the amount of porphobilinogen formed at 37 ºC. The reaction was started by addition of the substrate (ALA) into blood or tissue homogenates samples and incubated for 90 min (blood) or 60 min (liver and brain homogenates). The reaction product (porphobilinogen) was determined using modified Ehrlich's reagents and the absorbance was measured at 555 nm.

2.5 Non-protein thiol groups (NPSH) determination

Non-protein thiol groups (NPSH) were determined as described by Ellman (1959) with slight modifications. NPSH compounds were measured in the supernatant of hemolised blood, which was obtained after protein precipitation with 1 volume of 10% trichloroacetic acid followed by centrifugation. An aliquot of supernatants was added to 800 mmol/L phosphate buffer, pH 7.4, and 500 µmol/L DTNB (5,5'-dithio-bis-2-nitrobenzoic acid). Color development resulting from the reaction between DTNB and thiols reached a maximum in 5 min and was stable for

more than 30 min. Absorbance was read at 412 nm after 10 min. A standard curve of reduced glutathione was used in order to calculate the NPSH groups in the samples.

2.6 Reactive species measurement

To estimate the level of reactive species (RS) production in the liver, brain and blood homogenates and whole blood were diluted (1:10 v/v) in 10 mM Tris/HCl (pH 7.4) and incubated with 10 µmol/L of dichlorofluorescein diacetate (DCF-DA). The reactive species levels were determined by a spectrofluorimetric method, using 2′,7′-dichlorofluorescein diacetate (DCHF-DA) assay. The oxidation of DCHF-DA to fluorescent dichlorofluorescein was measured for the detection of intracellular RS. The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) 10 min after the addition of DCHF-DA to the medium (Bonini et al., 2006).

2.7 Determination of Thiobarbituric Acid Reactive Substances (TBARS) levels

TBARS were determined in the tissue homogenates and serum by the method of Ohkawa et al. (1979), in which malondialdehide (MDA), an end-product of lipid peroxidation, reacts with thiobarbituric acid (TBA) to form a colored complex. In brief, samples were incubated at 100 ℃ for 60 min in acid medium containing 0.45% sodium dodecyl sulfate and 0.6% thiobarbituric acid. After centrifugation, the reaction product was determined at 532 nm using 1,1,3,3- tetramethoxypropane as standard and the results were expressed as nmol MDA/mL serum or nmol MDA/g wet tissue.

2.8 Copper ion-induced lipid peroxidation of the liver homogenate

Cooper sulphate ($CuSO_4$) was added to an aliquot of liver homogenate at the final concentration of 10 µmol/L and incubated at 37 °C with continuous shaking. The peroxidation level after 1 h incubation was monitored by the measurement of TBARS as described above.

2.9 Ascorbic acid determination

Ascorbic acid determination was performed in the tissue homogenates as described by Jacques-Silva et al. (2001). Supernatants were precipitated with 1 volume of a cold 10% trichloroacetic acid solution followed by centrifugation. An aliquot of 300 µL of supernatants was mixed with 2,4-dinitrophenylhydrazine (4.5 mg/mL), $CuSO_4$ (0.075 mg/mL) and trichloroacetic acid 1.5 % in a final volume of 1 mL, and incubated for 3 h at 37º C. Then, 1 mL of H_2SO_4 65 % (v/v) was added to

the medium. The content of ascorbic acid was calculated using a standard curve (1.5-4.5 µmol/L ascorbic acid freshly prepared in sulfuric acid) and expressed as µmol ascorbic acid/g wet tissue.

2.10 Statistical analysis

Data are expressed as means ± SEM. Repeated measure ANOVA was used for statistical analysis of blood data. Statistical analysis was performed using a two-way analysis of variance (ANOVA), followed by Duncan's multiple range test when appropriate. Values of $p < 0.05$ were considered significant.

3 Results

The body weights of animals were measured once a week. Intake of the diet containing cholesterol (1%) and/or 10 ppm $(PhSe)_2$ did not change the body weight gain nor the final body weight of the rabbits (results not shown). As has already been reported (Bem et al., 2006; 2007), the consumption of dyphenyl diselenide for long time did not shown any apparent toxicity to rabbits.

3.1 Serum lipids and markers of oxidative stress in blood

Administration of cholesterol-rich diet to rabbits markedly increased total cholesterol serum levels after 25 and 45 days of treatment (approximately 8 and 12-fold, respectively) when compared with control group ($p<0.001$; Fig. 1A). On the other hand, $(PhSe)_2$ caused a significant decrease in total cholesterol levels in Chol/DD group ($p<0.01$). Such a hypocholesterolemic effect of $(PhSe)_2$ was evident after 45 days of treatment, when serum cholesterol levels were about 50% lower than that of Chol group ($p < 0.05$; Fig. 1A). Triglycerides and glucose serum levels were not modified by the different treatments (results not shown).

Intake of cholesterol-enriched diet by rabbits during 45 days increased significantly the production of serum TBARS and whole blood reactive species ($p<0.05$; Fig 1B; C). In contrast, the consumption of $(PhSe)_2$ caused a significant decrease in the production of serum TBARS and whole blood reactive species in Chol/DD group ($p < 0.05$). The serum TBARS levels of Chol/DD rabbits were about 2-fold lower than that of Chol group and similar to control group (Fig 1B; C).

Blood δ-ALA-D activity of hypercholesterolemic rabbits (Chol group) increased significantly after 25 days of treatment but returned to the control level after 45 days

($p<0,05$; Fig 1D). On the other hand, simultaneous intake of $(PhSe)_2$ and cholesterol were not associated with an increase in δ-ALA-D activity 25 days. The consumption of $(PhSe)_2$ by control rabbits (DD group) also increased the blood δ-ALA-D activity after 25 and 45 days ($p<0.05$; Fig 1D).

3.2 Measurement of tissue oxidative stress

3.2.1 Tissue TBARS levels

The effects of cholesterol- and/or $(PhSe)_2$-supplemented diets on lipid peroxidation in liver and brain, as measured by the TBARS content, are shown in the Figure 2. Feeding cholesterol-rich diet promoted a 7% increase in the hepatic TBARS levels of Chol rabbits (Fig. 2A), however this difference did not reach statistical significance. Nevertheless, the hepatic TBARS content was 13% lower in the Chol/DD group when compared to Chol group ($p<0.05$). In contrast, $(PhSe)_2$ alone did not change the hepatic TBARS levels of DD rabbits in comparison to control animals (Fig. 2A). On the other hand, no statistical differences were found in the brain TBARS content of rabbits regardless of treatments (Fig. 2B).

3.2.2 Effect of $(PhSe)_2$ on ex vivo Cu^{2+}-induced lipid peroxidation of liver

Figure 3 shows TBARS levels in the liver of all groups after 1 h incubation of hepatic homogenate with copper ion. Feeding of high cholesterol diet enhanced *ex vivo* copper ion-induced liver lipid peroxidation and the intake of $(PhSe)_2$ inhibited this oxidative susceptibility ($p<0.05$). No significant difference was observed in the TBARS levels between DD and control group. Similar results were also found using ferrous iron as oxidant (results not shown).

3.2.3 Reactive species measurement

Cholesterol and/or $(PhSe)_2$ ingestion did not change hepatic reactive species production (Fig. 4A). Treatment with cholesterol increased significantly the production of reactive species in brain, as shown by the high level of fluorescence intensity (Fig. 4B). $(PhSe)_2$ was effective in limiting reactive species enhancement caused by cholesterol in rabbit brain ($p < 0.05$) (Fig 4B).

3.3 δ-ALA-D activity

Cholesterol-rich diet significantly increased hepatic *δ-ALA* activity in Chol rabbit group ($p < 0.05$, Fig. 5A). A similar enhancement of the hepatic enzyme activity

was seen in groups treated with $(PhSe)_2$ (DD group) and in the Chol/DD group ($p<0.05$). In brain, cholesterol intake was not associated with an increase in *δ-ALA-D* activity, but $(PhSe)_2$ consumption (regardless of simultaneous cholesterol intake) was associated with an increase in cerebral *δ-ALA-D* ($p < 0.05$; Fig. 5B).

3.4 Non-protein–SH measurement

The cholesterol-enriched diet decreased hepatic NPSH content by 15% but this difference did not reach statistical significance (Fig. 6A). On the other hand, the treatment with $(PhSe)_2$ increased NPSH levels in rabbit liver of DD and Chol/DD groups ($p<0.05$). However, there was no alteration in NPSH levels in brain after cholesterol and/or $(PhSe)_2$ supplementation (Fig. 6B).

3.5 Ascorbic acid levels

Treatment of rabbits with $(PhSe)_2$ was associated with a significant decrease of hepatic ascorbic acid levels (Fig. 7A), regardless of cholesterol in the diet ($p<0.05$). However, the cholesterol and/or $(PhSe)_2$ treatments did not change the levels of ascorbic acid in the brain (Fig. 7B).

4 Discussion

$(PhSe)_2$ is a simple diorganoilselenium compound, which has been demonstrated to exhibit neuroprotective and antioxidant activities *in vitro* (Rossato et al., 2002 ; Meotti et al., 2004 ; Posser et al., 2006; Puntel, 2007) and *in vivo* (Ghisleni et al., 2003; Burger et al., 2004 ; Borges et al. 2006, Barbosa et al., 2006). In this study, we have evaluated the hypolipidemic and antioxidant effects of $(PhSe)_2$ on blood, liver and brain of rabbits submitted to a high-cholesterol diet. Our results provide evidences that oral administration of $(PhSe)_2$ had beneficial effects on serum total cholesterol and several parameters related to oxidative stress, ameliorating the oxidative status of hypercholesterolemic rabbits.

Feeding rabbits with a cholesterol-rich diet has been shown to increase oxidative stress and lipid peroxidation (Mahfouz and Kummerow, 2000). It is well documented that oxidative stress is one of the factors that links hypercholesterolemia to atherosclerosis (Harrison et al., 2003) and the addition of antioxidants to a cholesterol-supplemented diet has been shown to significantly reduce the severity of atherosclerotic lesions (Wojcicki, et al., 1991, Negis et al, 2006; Jenner et al, 2007).

As expected, we found that serum levels of total cholesterol increased significantly in rabbits receiving a cholesterol-rich diet, however, total cholesterol levels decreased about 50% in rabbits receiving the same hypercholesterolemic diet supplemented with $(PhSe)_2$ ($p<0.05$; Fig. 1A). To our knowledge, these results suggest, for the first time, that $(PhSe)_2$ can be effective at reducing serum cholesterol (Fig 1A).

The decrease in serum cholesterol may be due to several mechanisms, such as an inhibition of endogenous synthesis of cholesterol and/or apolipoprotein B, an increase in the plasma lipoprotein clearance rate and/or a decreased intestinal absorption of diet and bile cholesterol. The mechanism by which $(PhSe)_2$ diminished the hypercholesterolemia has not been investigated in the current study. However, Dhingra and collaborators (2006) demonstrated that selenium supplementation in the diet had a protective effect against hypercholesterolemia in rats, i.e., decreasing the serum levels of total cholesterol and apolipoprotein B-100 due to a reduction of HMG-CoA reductase mRNA expression and a probable increase of the expression of hepatic LDL receptor. Therefore, whether hypocholesterolemic effect of $(PhSe)_2$ has a similar mechanism of action needs further research.

Furthermore, we observed an increase in the serum levels of lipid peroxidation (TBARS) and an enhanced production of blood reactive species in hipercholesterolemic rabbits (Fig. 1). $(PhSe)_2$ significantly reduced these markers of oxidative stress (Fig 1B). Furthermore, *in vitro* and *ex vivo* studies have also demonstrated that $(PhSe)_2$ is a potential antioxidant compound (Rossato et al., 2002; Meotti et al., 2004; Ghisleni et al., 2003; Posser et al., 2006). Therefore, the beneficial effect of $(PhSe)_2$ to cholesterol-fed rabbits appear to be mediated by its lipid-lowering capacity and its antioxidant properties.

The antioxidant potential of $(PhSe)_2$ can be explained in part based on its glutathione peroxidase-like activity. In fact, the reaction catalyzed by organoselenium compound is similar to that catalyzed by glutathione peroxidase (Nogueira et al., 2004) and is of particular significance for living cells because it decomposes hydrogen peroxide, an intermediate that can give origin to the extremely reactive and toxic product $^{\bullet}OH$ (Draper and Hadley, 1990).

The consumption of a cholesterol-rich diet by rabbits did not affect the levels of hepatic and cerebral lipid peroxidation, as measured by the TBARS content (Fig. 2). Nevertheless, in this study we showed that hepatic TBARS content from Chol/DD group was significantly lower than that of Chol group (Fig. 2). Furthermore, the result

of *ex vivo* Cu^{2+}-induced lipid peroxidation of liver indicated that a high cholesterol diet increases the susceptibility of liver to lipid peroxidation and simultaneous ingestion of $(PhSe)_2$ abolished it (Fig. 3). This antioxidant effect are in accordance with previous studies of our group showing that $(PhSe)_2$ are effective antioxidant against TBARS production induced by different pro-oxidant agents (Rossato et al., 2002; Posser et al., 2006; Puntel et al., 2007). Additionally, we demonstrated recently that $(PhSe)_2$ was also a potent *in vitro* inhibitor of human LDL oxidation (Bem et al., submitted).

δ-ALA-D is extremely sensitive to the presence of pro-oxidants elements, which can oxidize its –SH groups during the oxidative stress (Perottoni et al., 2004). In contrast to these studies, here we observed that cholesterol increased δ-ALA-D activity in liver. Recent points of evidence from studies with rabbits, demonstrated that $(PhSe)_2$ also increased hepatic and cerebral δ-ALA-D activities and hepatic NPSH levels after a long term exposure (Bem et al., 2007). Similarly, the results of the present study indicated that the increase in hepatic *δ*-ALA-D in $(PhSe)_2$-supplemented groups may be related to an increase in NPSH found in this tissue (Fig. 6). In fact, it has been shown that *δ*-ALA-D activity might be modulated by the thiol status, and this phenomenon appears to be related to the presence of sulfhydryl groups located at the active center of the enzyme (Barnard et al., 1977).

Vitamin C is a well-known marker of oxidative stress and its reduction has been associate to an increase in oxidative stress. Here, we observed a decrease in vitamin C levels in the liver of rabbits treated with $(PhSe)_2$, but not in brain (Fig. 7). These results confirm our previous studies which demonstrated that high doses of $(PhSe)_2$ for a long period of time decreased vitamin C content in blood and liver of rabbits (Bem et al. 2006; 2007). However, TBARS levels, an indirect indicator of lipid peroxidation, and reactive species production were not altered in blood and in liver after ingestion of $(PhSe)_2$. Altogether, these results indicate that such decrease in vitamin C levels was not accompanied by an increase in the oxidative status.

Our results suggest an association between the observed changes in blood, liver and brain biochemical parameters, notably oxidative stress, due to hypercholesterolemia and the beneficial effect of $(PhSe)_2$ ameliorating the oxidative status mediated by a high cholesterol diet. Additionally, $(PhSe)_2$ was effective in reducing hypercholesterolemia to a significant extent in cholesterol-fed rabbits.

Therefore, further researches are needed to investigate the mechanism(s) of action as well the efficacy of $(PhSe)_2$ as anti-atherogenic agent.

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Figures

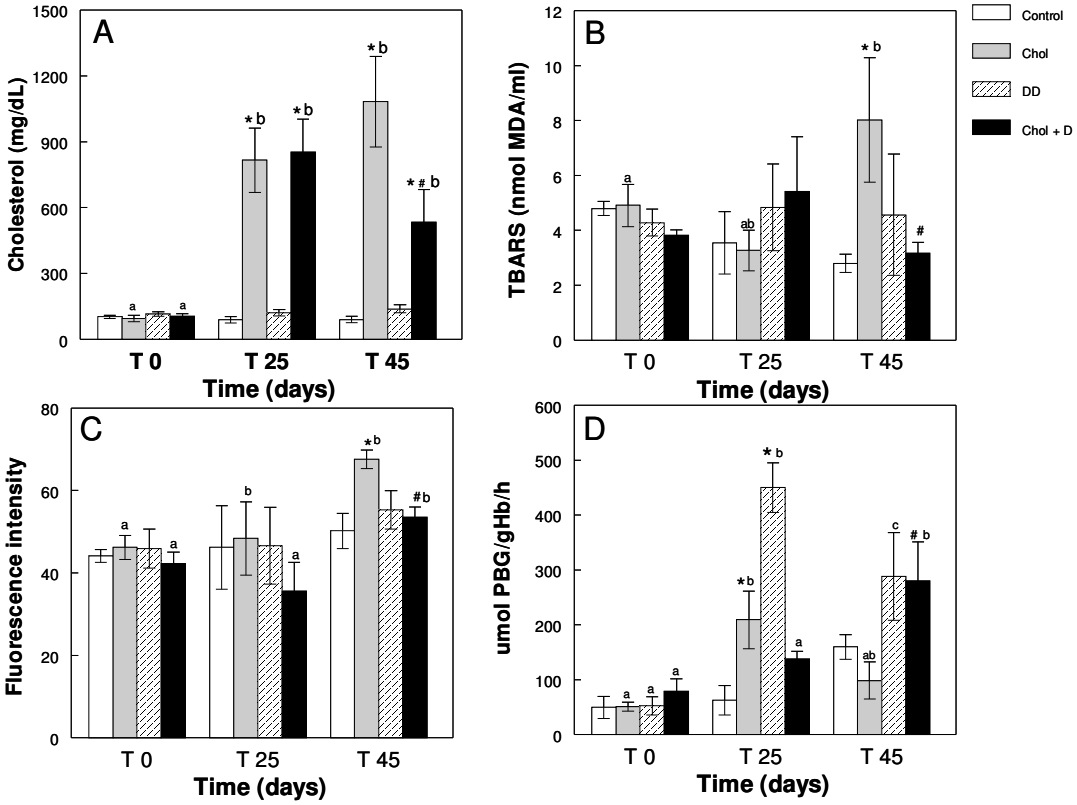

Figure 1. Effect of cholesterol and/or $(PhSe)_2$ intake on levels of (A) cholesterol and (B) TBARS in serum and (C) reactive species production and (D) δ-ALA-D activity in whole blood in rabbits. Data are expressed as means ± S.E.M. of six animals per group. (*) Denotes $p < 0.05$ as compared to control group. (#) Denotes $p < 0.05$ as compared to cholesterol group. (two-way ANOVA/Duncan). Means not sharing the same superscript letters (time effect) were different at $p < 0.05$ (repeated measures ANOVA/Duncan).

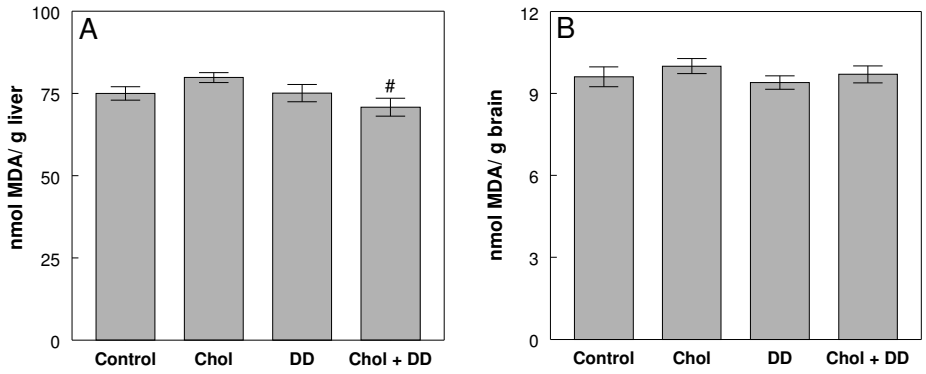

Figure 2. Effect of cholesterol and/or $(PhSe)_2$ intake on hepatic (A) and cerebral (B) TBARS levels in rabbits. Data are expressed as means ± S.E.M. of six animals per group. (#) Denotes $p < 0.05$ as compared to cholesterol group. (two-way ANOVA/Duncan).

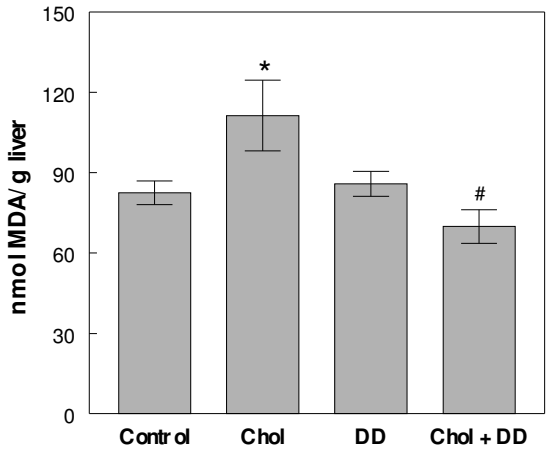

Figure 3. Effect of cholesterol and/or $(PhSe)_2$ intake on hepatic TBARS production after oxidation for 1 h at 37 ℃ with 10 μM CuSO4 in rabbits. Data are expressed as means ± S.E.M. of six animals per group. (*) Denotes $p < 0.05$ as compared to control group. (#) Denotes $p < 0.05$ as compared to cholesterol group. (two-way ANOVA/Duncan).

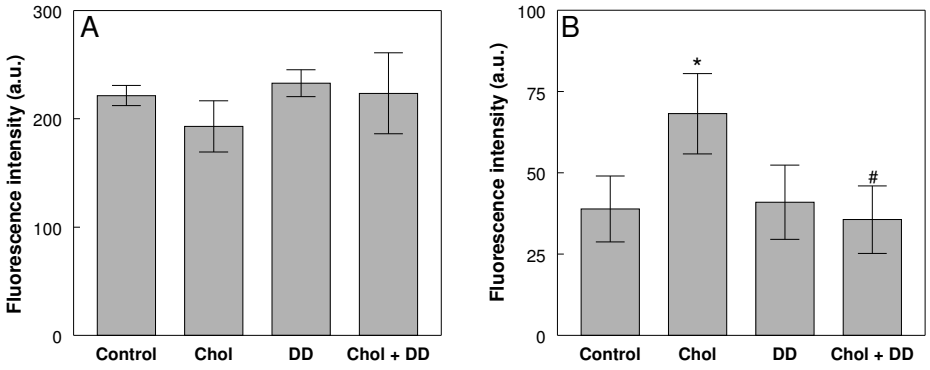

Figure 4. Effect of cholesterol and/or $(PhSe)_2$ intake on (A) hepatic and (B) cerebral reactive species production in rabbits. Data are expressed as means ± S.E.M. of six animals per group. (*) Denotes $p < 0.05$ as compared to control group (two-way ANOVA/Duncan).

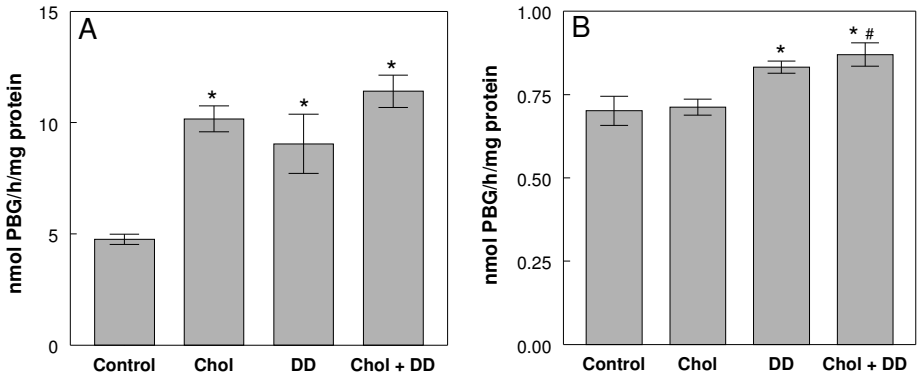

Figure 5. Effect of cholesterol and/or $(PhSe)_2$ intake on (A) hepatic and (B) cerebral δ-ALA-D activity in rabbits. Data are expressed as means ± S.E.M. of six animals per group. (*) Denotes $p < 0.05$ as compared to control group. (#) Denotes $p < 0.05$ as compared to cholesterol group. (two-way ANOVA/Duncan).

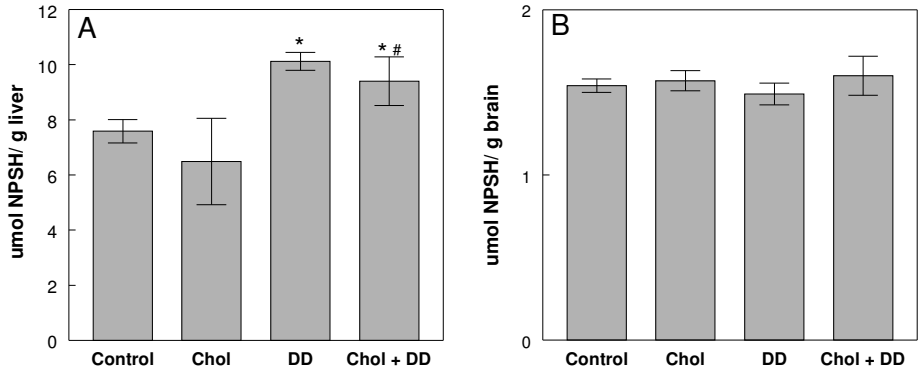

Figure 6. Effect of cholesterol and/or $(PhSe)_2$ intake on (A) hepatic and (B) cerebral NPSH levels in rabbits. Data are expressed as means ± S.E.M. of six animals per group. (*) Denotes $p < 0.05$ as compared to control group. (#) Denotes $p < 0.05$ as compared to cholesterol group. (two-way ANOVA/Duncan).

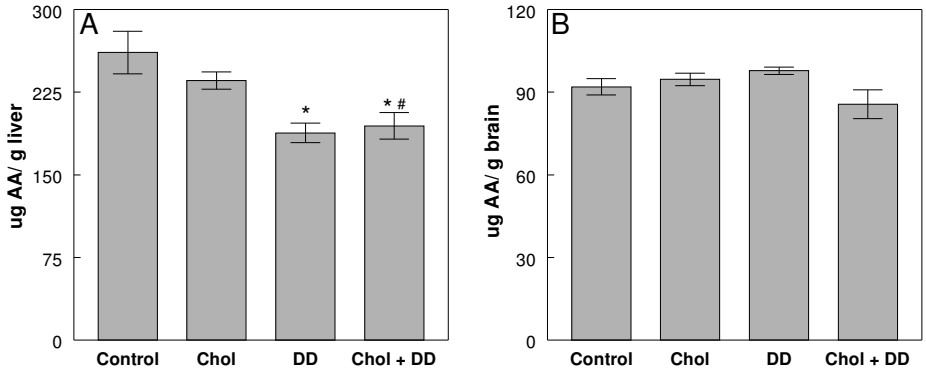

Figure 7. Effect of cholesterol and/or $(PhSe)_2$ intake on (A) hepatic and (B) cerebral ascorbic acid levels in rabbits. Data are expressed as means ± S.E.M. of six animals per group. (*) Denotes $p < 0.05$ as compared to control group. (#) Denotes $p < 0.05$ as compared to cholesterol group. (two-way ANOVA/Duncan).

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3.2 Estudos *in vitro*

3.2.1 Efeito do $(PhSe)_2$ na proteção contra à oxidação da LDL humana isolada.

3.2.1.1 Artigo 4

Diphenyl diselenide, a simple glutathione peroxidase mimetic, inhibits human LDL oxidation in vitro

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Diphenyl diselenide, a simple glutathione peroxidase mimetic, inhibits human LDL oxidation *in vitro*

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Abstract

Oxidative modification of low-density lipoprotein (LDL) represents an important factor in atherogenesis. In the present study, we have investigated the antioxidant capability of diphenyl diselenide $(PhSe)_2$, a simple organoseleno compound, against copper (Cu^{2+}) and peroxyl radical-induced human LDL oxidation *in vitro*. In initial studies using human serum, $(PhSe)_2$ caused a dose-dependent inhibition of Cu^{2+}-induced lipid peroxidation, which was correlated to thiol consumption. $(PhSe)_2$ increased lipid peroxidation *lag phase* and decreased lipid peroxidation *rate* in isolated human LDL, evaluated by measuring both conjugated diene and thiobarbituric acid reactive substances (TBARS) levels. Consistent with these observations, $(PhSe)_2$ showed a marked inhibitory effect on 2,2-azobis(2-amidinopropane dihydrochloride (AAPH)-induced oxidation of LDL or parinaric acid (PnA) incorporated into LDL. $(PhSe)_2$ also displayed a dose-dependent protective effect against Cu^{2+}-induced lipid peroxidation in rat aortic slices. Interestingly, besides the antioxidant effects of $(PhSe)_2$ toward the lipid moieties of LDL, which was related to its thiol-peroxidase activity, protein moieties from human isolated LDL were also protected against Cu^{2+}-induced oxidation. The results presented herein are the first to show that (i) $(PhSe)_2$ inhibits lipid peroxidation in human isolated LDL *in vitro*, (ii) this phenomenon is related to its thiol-peroxidase activity, and (iii) this chalcogen also prevents the oxidation of protein moieties of human LDL. Taken together, such data render $(PhSe)_2$ a promising molecule for pharmacological studies with respects to the atherogenic process.

Key words: diphenyl diselenide, selenium, ebselen, glutathione peroxidase, LDL oxidation, atherosclerosis.

1 Introduction

Atherosclerosis remains the most common cause of death in industrialized countries. This affects the vascular wall and leads to coronary artery diseases and cerebrovascular accidents (stroke) [1]. There is increasing evidence that oxidative modifications of low density lipoprotein (LDL) play a pivotal role in the development of atherosclerosis [2]. Moreover, elevated levels of oxidized LDL have been positively correlated to the severity of acute coronary events [3] and have been considered a biochemical marker for coronary heart disease [4].

Although the molecular bases related to the triggering process of LDL oxidation remain unclear, the major mechanisms currently explored are metal ions dyshomeostasis [5], changes in lipoxygenase- and myeloperoxidase-related pathways [6], reactive oxygen and nitrogen species generation, and alterations in the thiol status [7]. Independently on how LDL oxidation is initiated, the subsequent biochemical events involve the loss of antioxidants and the production of lipid peroxides, which are decomposed to aldehydes and other products [8].

Since LDL oxidation plays a key role in the pathogenesis of atherosclerosis, antioxidants that can inhibit this oxidative process might be useful in preventing atherosclerosis-related pathological conditions, such as coronary artery diseases and stroke [9]. In this regard, it has been evidenced that antioxidant capability of LDL can be easily increased by dietary antioxidant supplementation [8]. In fact, many endogenous and exogenous compounds have been reported to display beneficial effects against LDL oxidation [10-12].

Selenium (Se) is an essential nutrient associated with the function of major metabolic pathways in the cell, where it is incorporated as selenocysteine at the active site of a wide range of proteins [13]. Selenium intake is inversely correlated to the incidence of atherosclerosis and coronary heart disease [14]. In endothelial cells, selenium can protect from oxidative damage by altering the expression of selenoproteins with antioxidant function, such as cytoplasmic glutathione peroxidase (cyGPX), phospholipid hydroperoxide glutathione peroxidase (PHGPx) and thioredoxin reductase (TR) [15-16].

The interest in organoselenium chemistry and biochemistry has increased in the last three decades mainly due to the fact that several organoselenium compounds (including Ebselen) possess antioxidant activity [17,18], which is

associated with its gluthatione-peroxidase like activity [19-24] and also with the fact that ebselen can be a substrate for thioredoxin reductase [25]. Of particular importance, Ebselen, has anti-inflammatory, antiatherosclerotic, and cytoprotective properties both in vitro and in vivo models [26,29]. Moreover, two clinical studies showed that ebselen improved the neurological outcome of acute ischemia stroke and subarachnoid hemorrhage [20,21], pointing to the potential therapeutic significance of organoselenides.

In addition to ebselen, diphenyl diselenide ($(PhSe)_2$), another organoselenium compound with antioxidant properties, has been reported to present lower toxicity than ebselen [24]. $(PhSe)_2$ caused minimal toxicity when administrated acutely to mice and rats [30] and after a long term exposure to rabbits [31,32]. Recently, studies from our laboratory have demonstrated that $(PhSe)_2$ caused a significant reduction in blood glucose and glycated proteins levels in diabetic rats [33]. Moreover, the in vitro and in vivo antioxidant potential of $(PhSe)_2$ was already demonstrated in several tissues and models, including brain, liver and platelets [24, 34-37].

Given the earlier observations, the present study was carried out to evaluate the potential beneficial effects of $(PhSe)_2$ in protecting human LDL against lipid and protein oxidation in vitro. The thiol-peroxidase capability of $(PhSe)_2$ was also evaluated in an attempt to delve into molecular mechanisms related to the aforementioned antioxidant effects.

2 Materials and Methods

2.1 Materials

Diphenyl diselenide $(PhSe)_2$ was synthesized according to a method previously described [38]. Analyses of the ^{1}H NMR and ^{13}C NMR spectra showed that the obtained compound had analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of $(PhSe)_2$ (99.9%) was determined by GC/HPLC. $(PhSe)_2$ was solubilized in ethanol PA and a 10 mM stock solution was stored at 4° C for less than 2 weeks. Immediately before use, $(PhSe)_2$ was diluted in ethanol PA at the required concentrations for the different assays. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.2 Serum oxidation

The study was approved by our Ethic Committee at Universidade Federal de Santa Maria. Blood samples were collected from healthy and normolipidemic volunteers after a 12-h overnight fasting period. Samples were left to clot in the dark at room temperature for 30 min and then centrifuged at 1500 g for 15 min. The serum was removed and immediately used in the oxidation assays and oxidations were performed in serum rather than in plasma to avoid potential interferences of substances such as EDTA, heparin, or citrate. Briefly, serum samples were diluted 1:4 in 10 mM phosphate buffer, pH 7.4 and incubated at 37 ºC with CuSO4 (100 µM) and/or $(PhSe)_2$ (0-200 µM). The total volume was 9 mL. At different timepoints, aliquots (200 µL) were removed for evaluating thiobarbituric acid reactive substances (TBARS) levels and consumption of the total thiol groups (-SH). Serum TBARS levels and thiol groups were determined based on Ohkawa and collaborators [39] and Ellman [40], respectively.

2.3 LDL isolation

LDL was isolated from fresh human plasma by discontinuous density-gradient ultracentrifugation as described by da Silva and collaborators [41], with slight modifications. Plasma of nonfasted healthy normolipidemic donors collected with EDTA (1 mg/mL) was pooled and sucrose (final concentration, 0.5%) was added to prevent LDL aggregation. Five milliliters of EDTA-plasma adjusted to a density of 1.22 g/mL with solid KBr (0.326 g/mL) was layered on the bottom of a centrifuge tube. Then 5 mL EDTA-containing sodium chloride solution (density 1.006 g/mL) was overlaid on top of the plasma. Ultracentrifugation was run at 65.000 rpm for 2 h at 4º C, in a Hitachi ultracentrifuge. LDL particles were collected by aspiration of the yellow band at the middle of the tube and dialyzed exhaustively overnight at 4º C against 6 L of 10 mM phosphate buffer to remove the excess salt and the majority of the EDTA. Protein concentration in LDL solution was determined by Lowry and collaborators [42]. The purity of LDL preparation was verified by agarose gel electrophoresis. Isolated LDL was stored at -20º C not longer than 2 weeks.

2.4 LDL oxidation

LDL oxidation was monitored by following the conjugated dienes (CD) formation [43] and the TBARS production [39]. LDL samples (50 µg protein/mL) were

pre-incubated at 37 °C in a medium containing 10 mM phosphate buffer, pH 7.4 and different concentrations of $(PhSe)_2$ (0-40 µM). After 10 minutes, $CuSO_4$ (1.6 µM, 5 µM or 10 µM) was added to the reaction medium and aliquots were removed at different timepoints for evaluating CD and TBARS production. In another set of experiments, $(PhSe)_2$ (40 µM) was added at different timepoints (0, 45 or 90 min) in an attempt to evaluate whether $(PhSe)_2$ could inhibit Cu^{2+}-induced LDL oxidation once the process was started.

Potential antioxidant effects of $(PhSe)_2$ against LDL oxidation were also evaluated after the addition of an azo initiator, 2,2-azobis(2-amidinopropane dihydrochloride (AAPH) (1 mM), whose slow decomposition allow for a constant rate of peroxyl radical formation for several hours [10, 44].

In the studies of CD formation, the temporal change in absorbance at 234 nm (which refers to the CD concentration [43]) is divided into three phases: a lag phase, a propagation phase, and a terminal phase. The lag phase was defined as the intercept of the tangent of the slope of the absorbance curve in propagation phase with the time axis, and was expressed in min. The rate of propagation was obtained from the slope of the absorbance curve during the propagation phase. Absorbance at 234 nm was normalized with respects to the absorbance at the beginning of the oxidation process.

2.5 Measurement of Parinaric acid fluorescence

Cis-Parinaric acid (PnA) (9,11,13,15-octadecatetraenoic acid) fluorescence was monitored in a Perkin-Elmer LS 50 spectrofluorometer provided with a thermostated cuvette containing a magnetic stirring device. The excitation and emission wavelengths were 324 and 413 nm, respectively (slit widths: 3.5 nm).

Preliminary studies were performed in order to determine the concentration of LDL and PnA that insure totally incorporation of the probe into LDL and a linear fluorescent response with PnA concentration. The assays were performed at 37 °C in 2 mL of phosphate buffer (110 mM NaCl, 20 mM phosphate, pH 7.4) containing 45 µg of LDL protein and an aliquot of ethanolic solution of PnA (1.5 µM final concentration). The incorporation of the probe was carried out by gentle stirring for 1 min. Increasing concentrations of $(PhSe)_2$ were added to the mixture and the oxidation reaction was initiated by addition of AAPH (10 mM final concentration). The

capacity of $(PhSe)_2$ to protect LDL from oxidation was determined by the inhibition of probe fluorescence decay [45].

2.6 Measurement of LDL-trp fluorescence

The fluorescence spectra of native LDL display a single band centered at approximately 332 nm, which is assigned to the Trp residues in apo B (Giessauf et al., 1995). Loss of tryptophan fluorescence is a marker for oxidations at the protein core of LDL [46,47]. Cu^{2+}-induced LDL oxidation was performed in a similarly to item 2.4, except that 3.3 µM $CuSO_4$, different $(PhSe)_2$ concentrations (0-30 µM) were used. Tryptophan fluorescence was measured at different timepoints (0-360 min) using a Shimatzo espectrofluorometer (excitation at 282 nm and emission at 331 nm).

2.7 Thiol Peroxidase Activity of $(PhSe)_2$ and Spectroscopy studies

The thiol-peroxidase activity of $(PhSe)_2$ was measured according to a method previously described by Pivetta and collaborators [48] and is a modification of the method described by Wilson and collaborators [19]. $(PhSe)_2$ (50 µM) was incubated at 37 ºC in a medium containing 50 mM potassium phosphate buffer, pH 7.0, 1 mM ethylene diamine tetraacetic acid (EDTA), 1 mM reduced glutathione (GSH), 1 U of GR and 0.25 mM NADPH (final volume of reaction = 1 mL). The reaction was initiated by addition of 0.5 µmol of hydrogen peroxide. The activity was followed by the decrease of NADPH absorption at 340 nm. Appropriate controls were carried out without $(PhSe)_2$ and were subtracted.

The catalytic effect of $(PhSe)_2$ on glutathione oxidation rate was assessed either in the presence or absence of hydrogen peroxide (H_2O_2). In short, different $(PhSe)_2$ concentrations (0-100 µM) were incubated at 37 ºC in a medium containing 10 mM phosphate buffer, (pH 7.4), 1.0 mM reduced glutathione and 0.2 mM H_2O_2. At different timepoints (0-15 min), aliquots of the reaction mixture (200 µL) were checked for the amount of free sulfhydryl groups [40].

In order to evaluate the potential chemical interaction between $(PhSe)_2$, GSH and H_2O_2, $(PhSe)_2$ (20 µM) was incubated with GSH (200 µM) in 10 mM phosphate buffer (pH 7.4). The reaction was performed at 37 ºC in a quartz cuvette and monitored spectrophotometrically (250–400 nm) using a Perkin-Elmer Lambda 6 spectrophotometer. In additional experiments, H_2O_2 (200 µM) was added 1 minute

after the reaction of $(PhSe)_2$ with GSH. The reaction mixture had 2 ml. Temperature was maintained using a circulating water bath. Readings were done against a reference cuvette containing phosphate buffer.

2.8 Aortic slices oxidation

Adult Wistar rats were decapitated, thoracic aorta were quickly dissected and then removed, rinsed and submerged in a petri dish filled with ice-cold isosmotic phosphate buffer (NaCl 124 mM, Na_2HPO_4 10 mM, NaH_2PO_4 5 mM, KH_2PO_4 5 mM, glucose 10 mM, pH 7.4) and cleaned of adherent fat and connective tissue in an ice-bath. Transverse propsections (400 µm) were prepared using a McIlwain tissue chopper. Aortic slices (10 slices) were incubated in isosmotic phosphate buffer with 20 or 40 µM of $(PhSe)_2$ and/or 10 µM $CuSO_4$ for two hours. After incubations, aorta slices were washed with saline and homogenized in 400 µl of acetic acid buffer (pH 3.5). Lipid oxidation in aorta homogenates was measured by determining thiobarbituric acid-reactive substances (TBARS), as described by Ohkawa and collaborators [39]. Experimental procedures involving animals were approved by the local Animal Care Committee.

2.9 Statistical analysis

Values are expressed as mean ± SEM. One-way analysis of variance (ANOVA) was used for multiple comparisons, followed by Duncan's multiple range test when appropriate. Values of $p < 0.05$ were considered significant. Linear regression analysis was performed to assess the association between two continuous variables. Statistical analyses were performed using Statistica 6.0 version software.

3 Results

3.1 Effects of $(PhSe)_2$ on serum oxidation

The protective effects of $(PhSe)_2$ against Cu^{2+}-induced TBARS generation and thiol consumption in blood serum are depicted in Figure 1. Cu^{2+} (100 µM) caused a time-dependent increase of serum TBARS levels and a time-dependent decrease of total thiol groups. Interestingly, even though $(PhSe)_2$ displayed an inhibitory effect toward Cu^{2+}-induced TBARS generation, there was a stimulation of thiol groups consumption by $(PhSe)_2$ (Figure 1).

3.2 Effects of $(PhSe)_2$ on the LDL oxidation

3.2.1 $(PhSe)_2$ effects on Cu^{2+}-induced LDL oxidation

The effects of $(PhSe)_2$ on Cu^{2+}-induced lipid oxidation in isolated LDL are depicted in Figure 2 and Table 1. $(PhSe)_2$ inhibited Cu^{2+}-induced generation of both conjugated dienes (Figure 2, left) and TBARS (Figure 2, right) in a concentration dependent manner. Cu^{2+} displayed concentration-dependent oxidative effects toward lipids, evaluated by measuring conjugated dienes (Figure 2; Table 1). LDL oxidation showed an expected oxidation pattern of an initial *lag phase* followed by a *propagation phase* and *a decomposition phase* (Figure 2, left), as originally described by Esterbauer and collaborators [43]. Interestingly, $(PhSe)_2$ caused concentration-dependent increases in *lag phase* and decreases in the *oxidation rate*, evidenced by changes in the propagation phase slope.

In another set of experiments, $(PhSe)_2$ (40 µM) was added at different timepoints (0, 45 or 90 min) in an attempt to evaluate whether $(PhSe)_2$ could inhibit Cu^{2+}-induced lipid LDL oxidation once the process was started. Figure 3 shows a significant inhibitory effect of $(PhSe)_2$ toward Cu^{2+}-induced lipid LDL oxidation when present at the beginning of the oxidation process. Interestingly, $(PhSe)_2$ inhibited Cu^{2+}-induced lipid LDL oxidation when added to the reaction medium at 45 and 90 min after the oxidation process was started.

3.2.2 $(PhSe)_2$ effects on AAPH-mediated lipid LDL oxidation

The effects of $(PhSe)_2$ on AAPH-mediated lipid LDL oxidation is depicted in Figure 4. AAPH, which generates hydroxyl radical at a constant rate in aqueous medium and induces the chain oxidation of human LDL by a free radical mediated mechanism [44], caused a significant increase of CD formation within time (Figure 4). $(PhSe)_2$ (3-50 µM) showed a marked inhibitory effect on AAPH-induced lipid LDL oxidation in a concentration dependent manner. In fact, a significant negative correlation ($r^2 = 0.9292$, $p < 0.001$) was observed for $(PhSe)_2$ concentrations and CD levels (Inset Figure 4).

3.2.3 $(PhSe)_2$ effects on parinaric acid fluorescence

PnA is a polyunsaturated fatty acid that has been used successfully as a fluorescent probe to monitor the initial stages of lipid peroxidation by peroxyl radicals from AAPH [45]. The inset of Figure 5 indicates a typical control assay where the

decrease of the fluorescence of PnA following the addition of AAPH reflects its oxidative degradation. Figure 5 shows that low concentrations of $(PhSe)_2$ (2, 3 and 4 µM) caused a concentration-dependent inhibition of the fluorescence intensity decay of PnA in the presence of AAPH when compared to the control condition (absence of $(PhSe)_2$).

3.3 Effects of $(PhSe)_2$ on the LDL tryptophan fluorescence emission

The time course of tryptophan (Trp) fluorescence emission intensity was used to monitor Cu^{2+}-induced apolipoprotein LDL oxidation. Figure 6 shows that protein moieties of LDL are oxidized within time in the presence of CuSO4 (3.36 µM). This phenomenon was prevented by $(PhSe)_2$ in a concentration-dependent manner. It is noteworthy that Cu^{2+}-induced apolipoprotein LDL oxidation was almost completely abolished in the presence of 20 and 30 µM of $(PhSe)_2$.

3.4 Aortic slices oxidation

The effects of $(PhSe)_2$ on Cu^{2+}-induced lipid peroxidation in rat aortic slices are depicted in Figure 7. Incubation of aortic rats slices with $(PhSe)_2$ for 1 hour was associated with a concentration-dependent reduction in lipid peroxidation induced by copper ions. One-way ANOVA showed that 10 µM Cu^{2+} increased ($p < 0.05$) lipid peroxidation in rat aortic slices. Co-incubation with $(PhSe)_2$ was effective in decreasing Cu^{2+}-induced TBARS formation in a concentration-dependent manner (Figure 7).

3.5 Spectroscopy studies

In order to delve into molecular mechanisms involved with the protective role of $(PhSe)_2$ against LDL lipid peroxidation, in vitro experiments of light/UV spectroscopy concerning the chemical interaction between $(PhSe)_2$, GSH and H_2O_2 were carried out. The unique spectral characteristic of $(PhSe)_2$ (20 µM, Figure 8, spectrogram A) was changed after the reaction with excess of GSH (200 µM), probably due to the formation of phenyl selenol intermediate ($PhSe^-$) (Figure 8, spectrogram C). Interestingly, after the addition of H_2O_2 (200 µM), the strong and broad absorption peak at 270 nm of such intermediate was abolished, indicating its chemical interaction with H_2O_2 (Figure 8, spectrogram D). Consistent with this observation, $(PhSe)_2$ displayed a concentration-dependent glutathione peroxidase-like activity (Figure 8, inset), which was indirectly measured by NADPH consumption

in the presence of glutathione, purified glutathione reductase and *hydrogen p*eroxide. The peroxidase-like activity of $(PhSe)_2$ was approximately twice when compared to that of ebselen (data not show), these results are in agreement with the previous study of Wilson and collaborators (1989), where they showed that $(PhSe)_2$ is about 1.6 more effective as GPx-mimetic than ebselen. Moreover, $(PhSe)_2$ catalytically increased glutathione oxidation in the presence of H_2O_2 in a concentration-dependent manner (Figure 9). Taken together, these data indicate that the chemical interaction between $(PhSe)_2$ and glutathione produces an intermediate that is capable of interacting with peroxides. This process allow for the detoxification of peroxides at the expenses of sulfhydryl groups from glutathione (Figure 8 and 9).

4 Discussion

Oxidative damage to lipoproteins, in particular low density lipoprotein (LDL), is known to play a role in a number of diseases associated with ageing and is in agreement with the oxidative stress theory of ageing [49]. Oxidized LDL is involved in the development of atherosclerosis through the formation of foam cells [50] and autoantibodies against oxidized LDL [51]. Since oxidative damage to LDL is involved in the development of atherosclerosis, several recent studies have sought for the potential beneficial effects of antioxidant molecules against LDL oxidation under in vitro [52-54] and in vivo [55,56] conditions. Of particular importance, epidemiological studies have pointed to red wine polyphenols as promising molecules that could prevent the development of several coronary syndromes by inhibiting the atherogenic process [57].

Here, we observed that $(PhSe)_2$, an organoseleno compound with antioxidant properties, displayed concentration-dependent protective effects against human LDL oxidation induced by both cuprous ion (Cu^{2+}) and 2,2-azobis(2-amidinopropane dihydrochloride (AAPH), a hydroxyl radical generator. It is noteworthy that $(PhSe)_2$ increased the oxidation lag phase and decreased the oxidation rate in human LDL and these phenomena were evidenced using different methodological approaches, such as the measurement of thiobarbituric acid reactive substances, conjugated dienes generation and parinaric acid (PnA) incorporated into the lipoprotein. Even though several studies have reported the beneficial effects of organoseleno compounds against pathological conditions associated to oxidative stress (inflammation, gastric mucosal damage, neurotoxicity, and hepatotoxicity [24]), there

are no studies in the scientific literature regarding potential beneficial effects of $(PhSe)_2$ against LDL oxidative damage. In fact, it appears that ebselen is the only organoseleno compound that has received considerable interest during the last decades [58-60]. In the present study, the choice for studding $(PhSe)_2$ as a potential beneficial molecule against human LDL oxidation was based on previous studies from our group, which have shown that $(PhSe)_2$ displays a higher capability of ebselen in detoxifying peroxides at expenses of sulfhydryl molecules [36].

From a molecular point of view, it is noteworthy that $(PhSe)_2$ prevented Cu^{2+}-induced peroxidation in human serum in a concentration-dependent manner, but this effects was correlated to the consumption of sulfhydryl groups, pointing to the involvement of a thiol peroxidase-like event. In agreement with this hypothesis, it was also observed that $(PhSe)_2$ has the capability to reduce hydrogen peroxide at expenses of reduced glutathione, indicating the versatility of $(PhSe)_2$ in detoxifying different types of peroxides. This idea is reinforced by the observed protective effects of $(PhSe)_2$ against the oxidation of endogenous and exogenous (incorporated PnA) LDL lipids. In agreement, spectroscopic studies showed the direct chemical interaction between $(PhSe)_2$ and glutathione, resulting in the formation of a chemical intermediary whose stability is affected by the presence of hydrogen peroxide.

An interesting effect of this study was that $(PhSe)_2$ displayed significant protective effects in the different phases of Cu^{2+}-induced LDL oxidation. In fact, when added to the reaction medium at time zero (Figure 3), $(PhSe)_2$ significantly prevented LDL oxidation. Moreover, when added to the reaction medium at 45 or 90 min (partially oxidized LDL), $(PhSe)_2$ was capable of stopping conjugate dienes formation. Mechanistically, these results are of interest because they indicate that the thiol-peroxidase activity of $(PhSe)_2$ is enough important to prevent the generation of secondary products of lipid peroxidation, such as conjugated dienes.

Another significant (and maybe the most important) result from our study was the capability of $(PhSe)_2$ to prevent Cu^{2+}-induced loss of tryptophan (Trp) fluorescence in human LDL. In this regard, it has been reported that the fluorescence spectrum of native LDL displays a single band centered at approximately 332 nm, which is assigned to the Trp residues in apo B (Giessauf et al., 1995) and loss of Trp fluorescence is a marker for oxidations at the protein core of LDL [46,47]. The protective effect of $(PhSe)_2$ against Cu^{2+}-induced loss of tryptophan fluorescence indicates that, besides it beneficial effects against oxidation of lipid moieties of LDL,

this chalcogen also prevents the oxidation of protein moieties of human LDL, pointing to an additional mechanism that could contribute to the inhibition of the atherogenic process.

The results of the present study are the first to show that (i) $(PhSe)_2$ inhibits lipid peroxidation in human isolated LDL in vitro, (ii) this phenomenon is related to its thiol-peroxidase activity, and (iii) this chalcogen also prevents the oxidation of protein moieties of human LDL. Taken together, such data render (PhSe)2 a promising molecule for pharmacological studies with respects to the atherogenic process.

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Figures

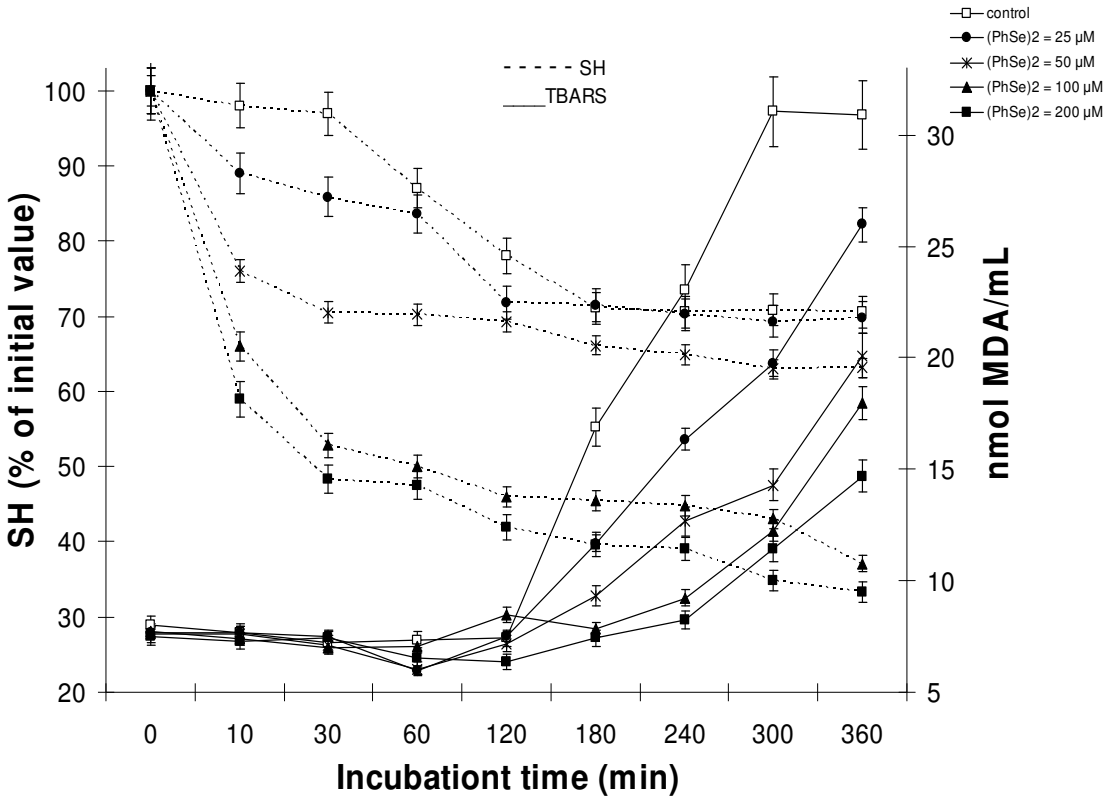

Figure 1 - Effects of $(PhSe)_2$ on Cu^{2+}-induced lipid peroxidation and thiol consumption in human serum. At different time points (indicated in the abscissa axis), aliquots were removed for analyzing TBARS (solids line) and SH (broken lines) content (see Materials and Methods Section). TBARS levels are expressed as nmol of malondialdehyde (MDA). Total thiol (-SH) content is expressed as percent of control, whose basal concentration was 437 ± 25 nmol/mL. Results are represented as mean ± standard error of mean (SEM) from at least three independent experiments.

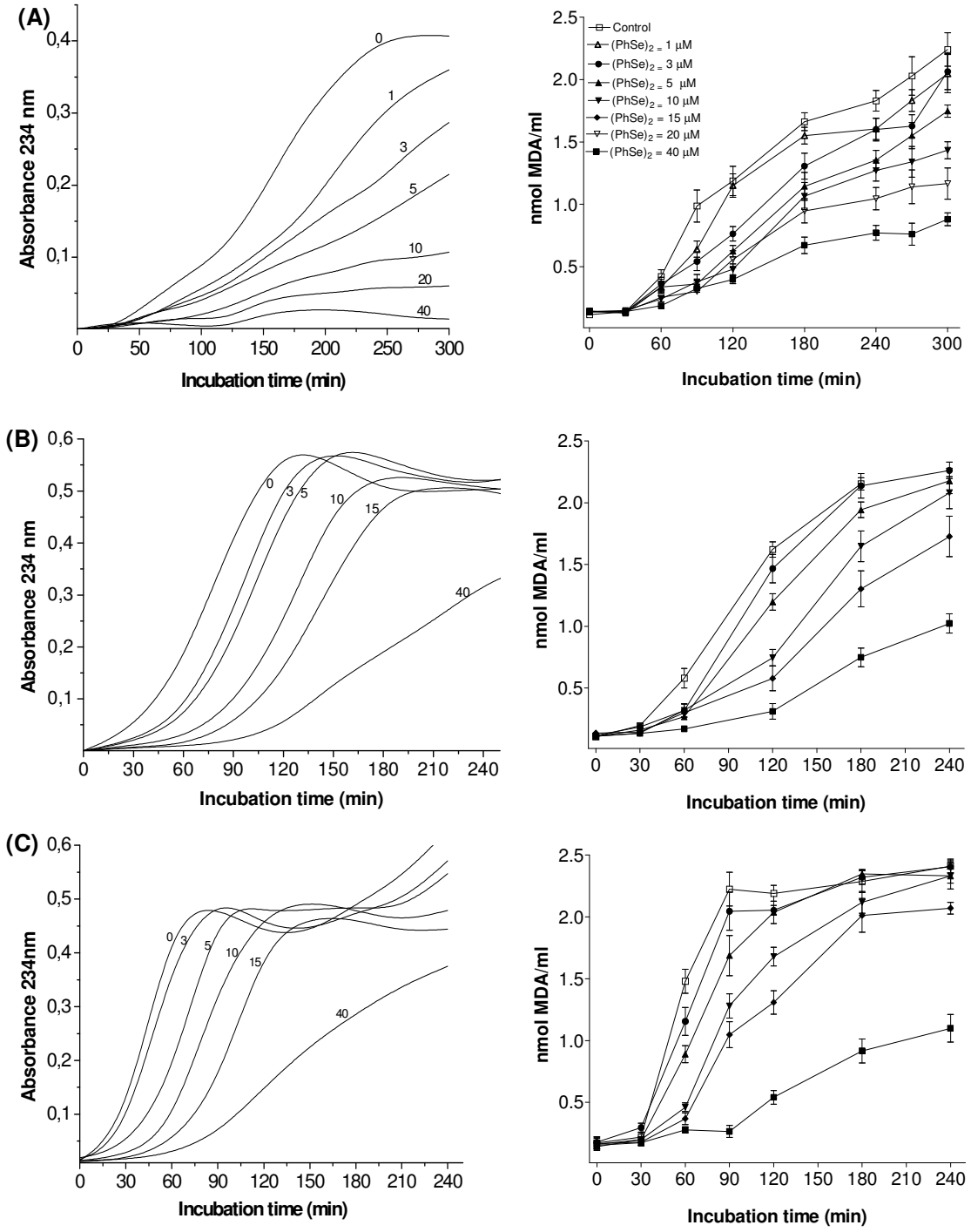

Figure 2 - Effects of $(PhSe)_2$ on Cu^{2+}-induced lipid peroxidation in human LDL. At different time points (indicated in the abscissa axis), aliquots were removed for analyzing conjugated dienes (left) and TBARS (right). LDL samples (50 µg protein/mL) were incubated in the presence of 1.6 µM (A), 5.0 µM (B) and 10 µM (C)

$CuSO_4$ and in the absence (control) or presence (1-40 µM) of $(PhSe)_2$. TBARS levels are expressed as nmol of malondialdehyde (MDA). Conjugated dienes are expressed as absorbance at 234 nm. Results are represented as mean ± standard error of mean (SEM) from at least three independent experiments.

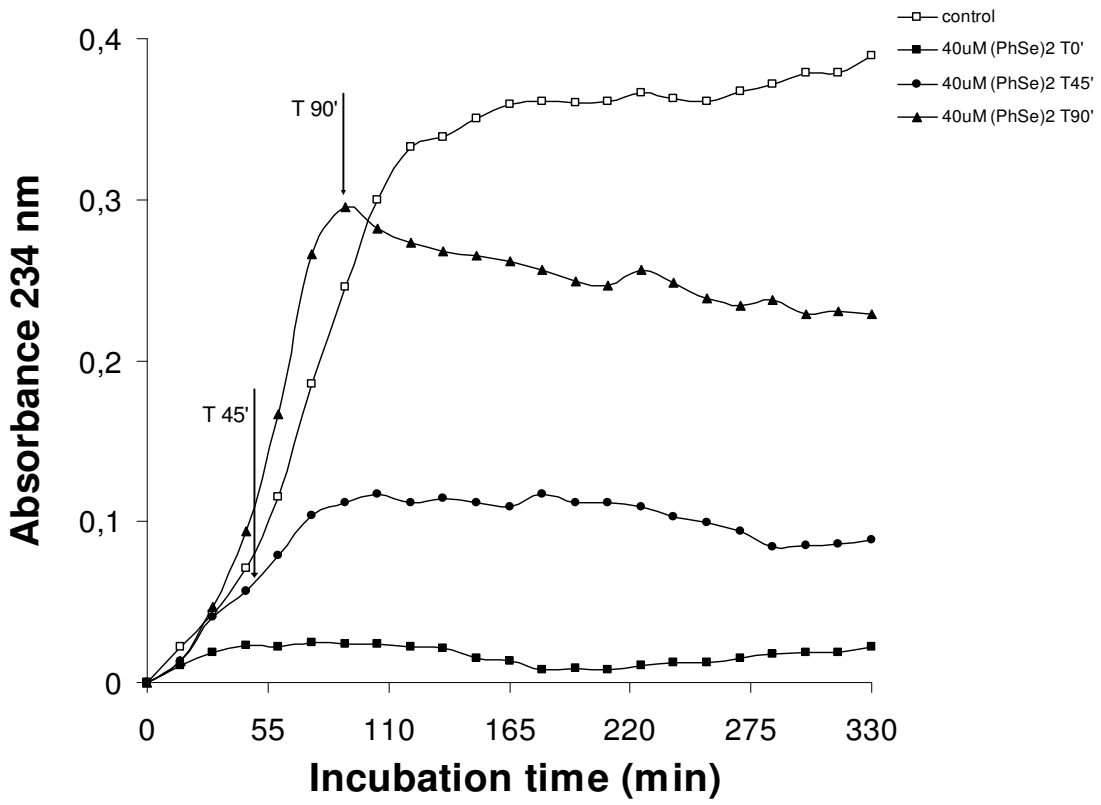

Figure 3 - Effects of $(PhSe)_2$ on Cu^{2+}-induced conjugated dienes formation in previously oxidized human LDL. LDL samples (50 µg protein/mL) were incubated at 37 °C in the presence of 1.6 µM $CuSO_4$ and in the absence (control) or presence (40 µM) of $(PhSe)_2$, which was added at 0, 45 or 90 min after $CuSO_4$ addition. At different time points (indicated in the abscissa axis), aliquots were removed for analyzing conjugated dienes. Results are expressed as absorbance at 234 nm. Results are derived from a single representative experiment. Experiments were repeated at least three times, showing similar results.

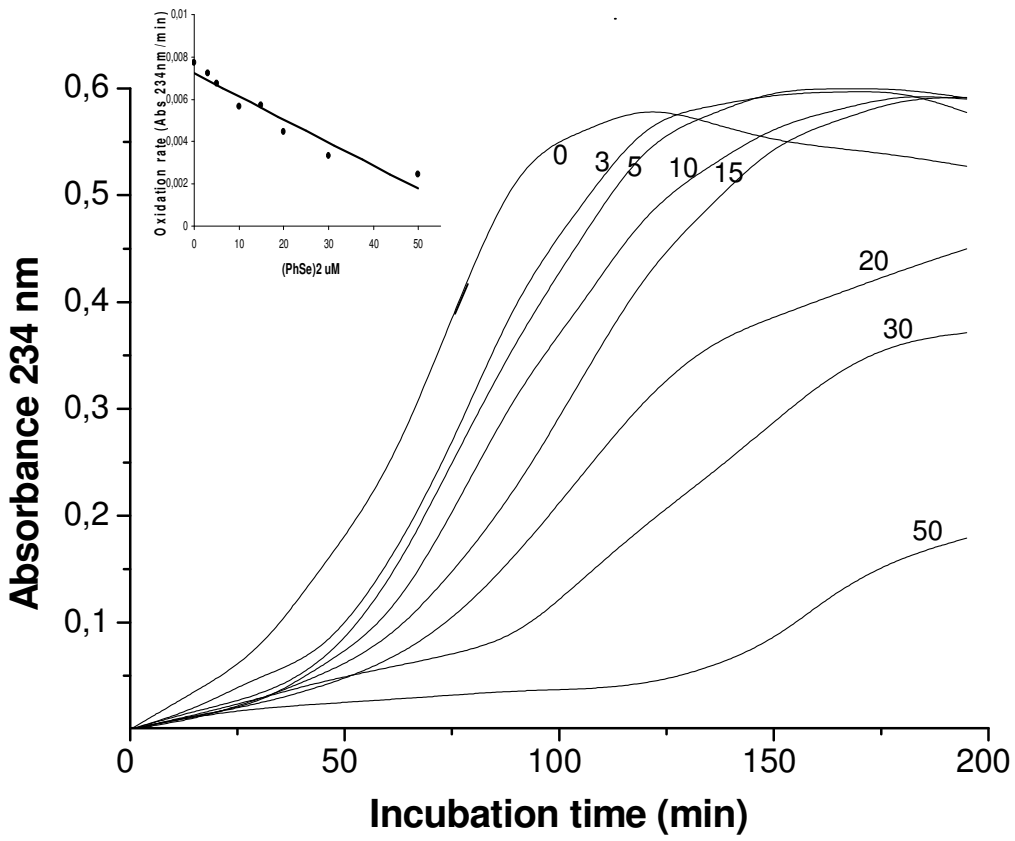

Figure 4 - Effects of $(PhSe)_2$ on AAPH-induced conjugated dienes formation in human LDL. At different time points, aliquots were removed for analyzing conjugated dienes. LDL samples (50 µg protein/mL) were incubated at 37 °C with 1.0 mM AAPH in the absence (control) or presence (3-50 µM) of $(PhSe)_2$. Conjugated dienes are expressed as absorbance at 234 nm. Results are derived from a single representative experiment. Experiments were repeated at least three times, showing similar results. Inset shows the significant correlation between oxidation rate and $(PhSe)_2$ concentrations.

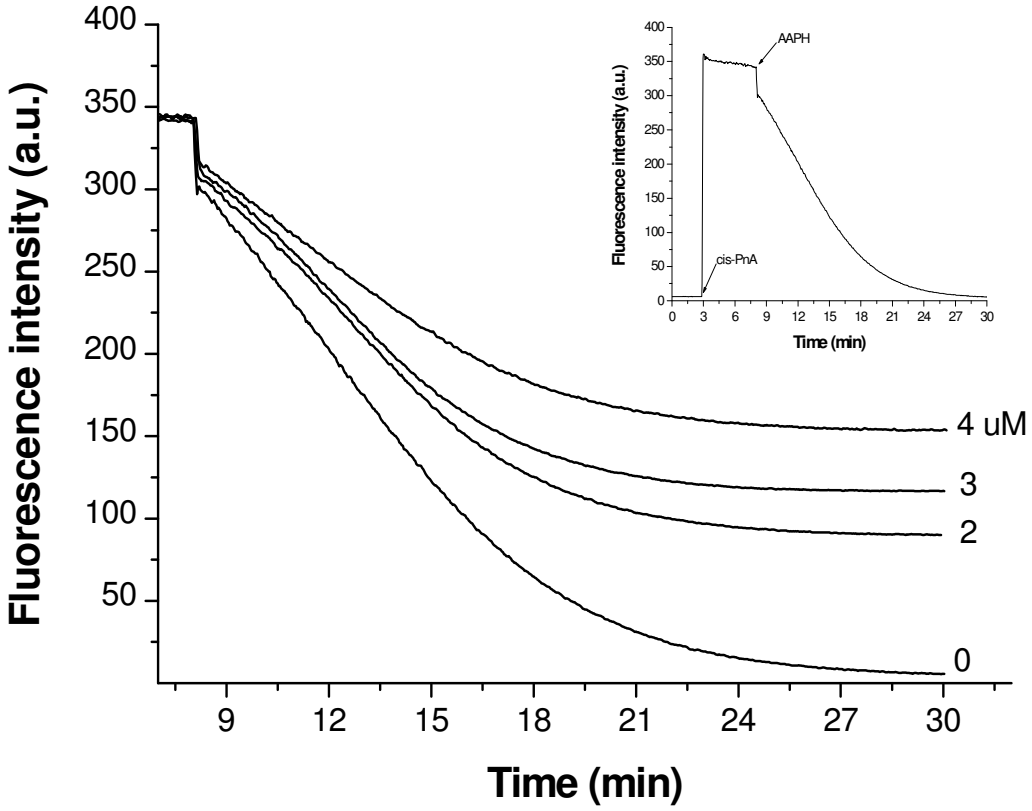

Figure 5 - Effect of $(PhSe)_2$ on AAPH-induced oxidation of parinaric acid (PnA) incorporated into LDL. LDL samples (45 µg protein) were incubated at 37 ºC with 1.5 µM PnA for 1 min under gentle stirring. AAPH (1.0 mM) and $(PhSe)_2$ (0, 2, 3, or 4 µM; three min after AAPH addition) were added to the reaction medium. Inset shows a typical control assay of PnA oxidation, incorporated into LDL, initiated by AAPH. The fluorescence intensity was recorded up to 30 min and shows the initial light scattering and the fluorescent signal decay following AAPH addition. Results are derived from a single representative experiment. Experiments were repeated at least three times, showing similar results.

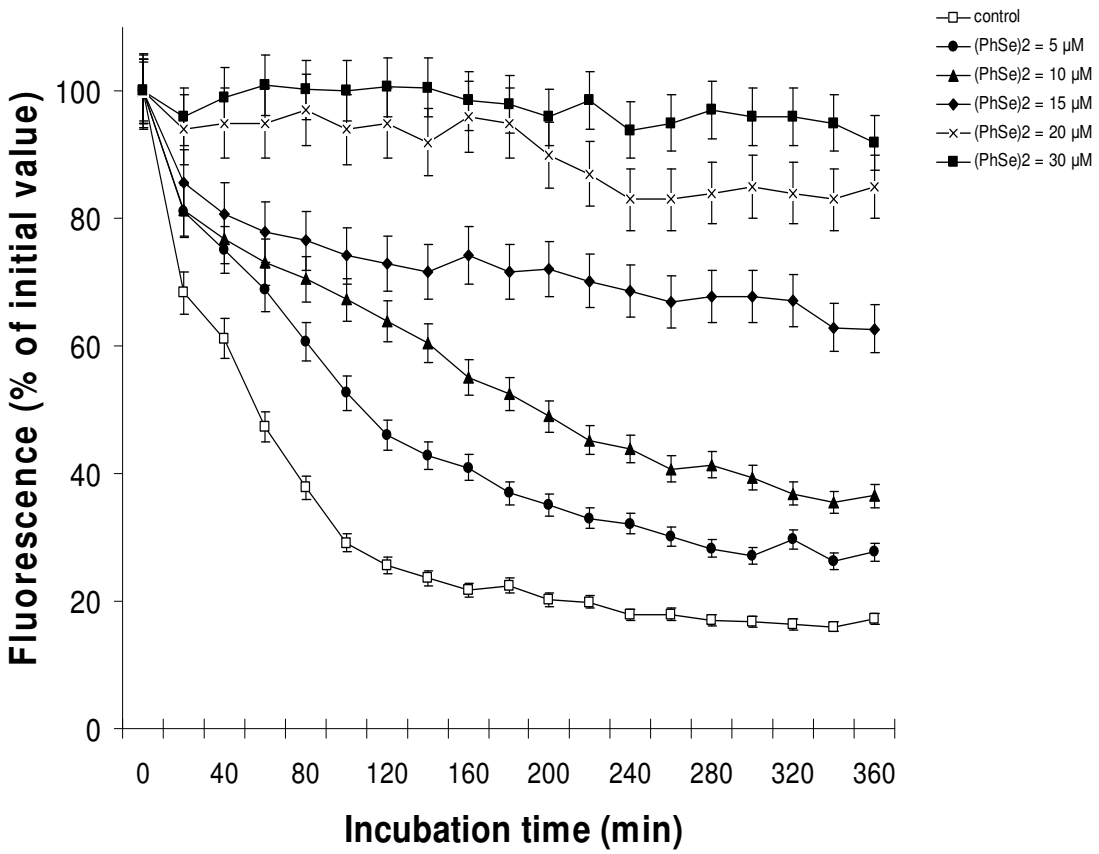

Figure 6 - Effects of $(PhSe)_2$ on Cu^{2+}-induced loss of tryptophan fluorescence in human LDL. LDL samples (50 µg protein/mL) were incubated at 37 ºC in the presence of 3.3 µM $CuSO_4$ and different $(PhSe)_2$ concentrations (0-30 µM). Tryptophan fluorescence (excitation at 282 nm and emission at 331 nm) was measured at different timepoints (0-360 min). Data are expressed as percentage of the emission intensity measured before Cu^{2+} addition. Results are represented as mean ± standard error of mean (SEM) from at least three independent experiments.

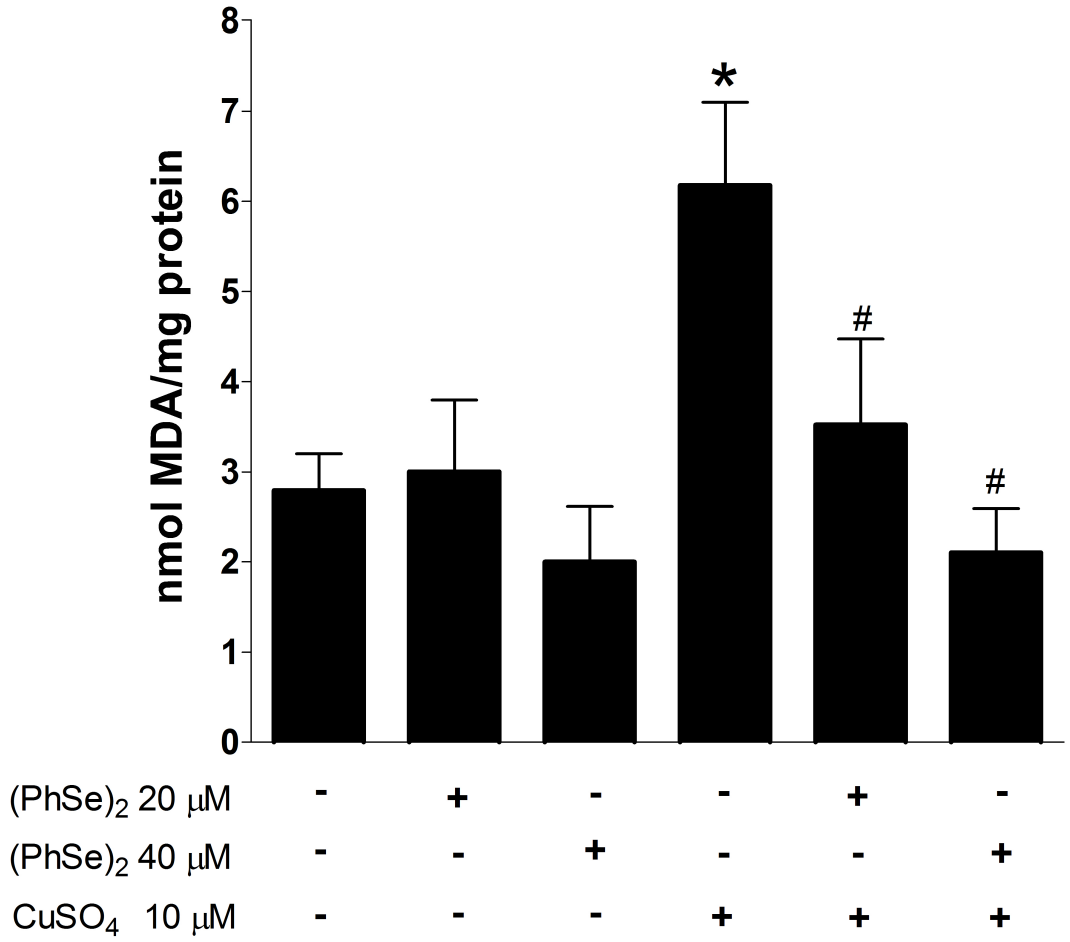

Figure 7 - Effect of $(PhSe)_2$ on copper induced lipoperoxidation in rat aortic slices. Data are expressed as nmol of malondialdehyde (MDA) per mg of protein and presented as mean ± standard error of mean (SEM) from at least three independent experiments. (*) Significantly different ($p < 0.05$) when compared to control group. (#)Significantly different ($p < 0.05$) when compared to 10 μM $CuSO_4$.

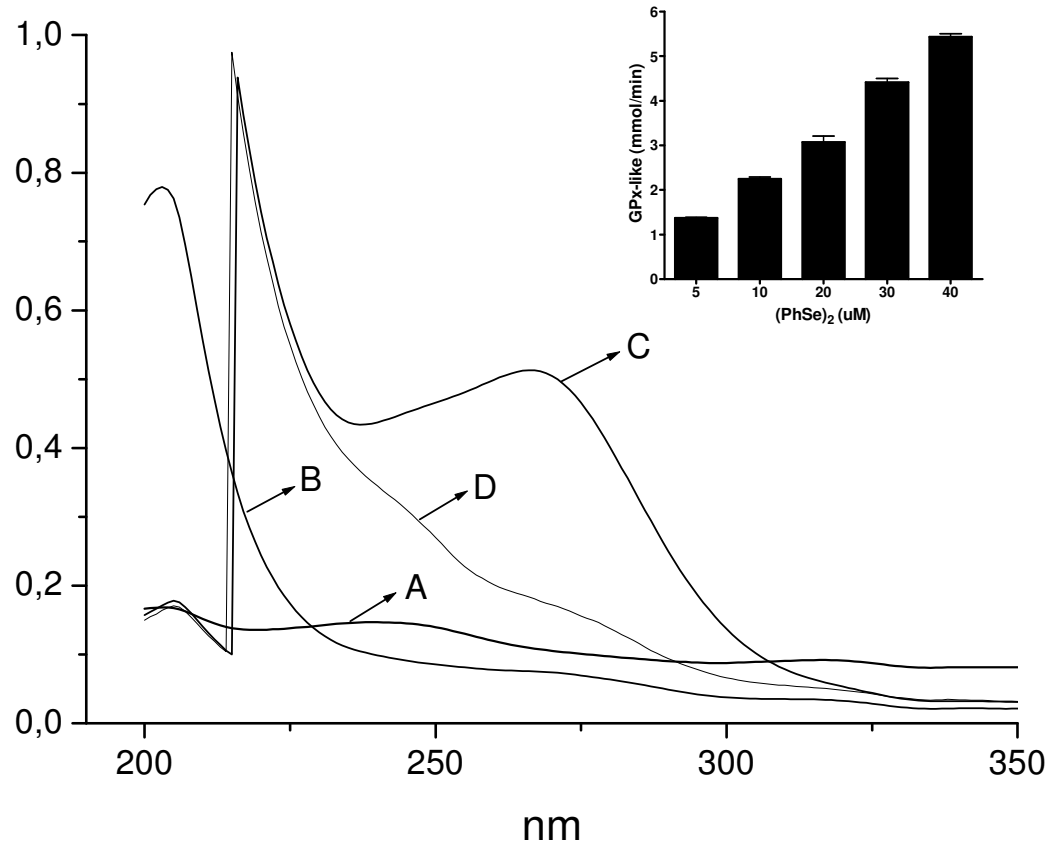

Figura 8 - The comparative spectra of $(PhSe)_2$ and the products of its interaction with GSH and/or H_2O_2. (A) $(PhSe)_2$ (20 µM); (B) GSH (200 µM); (C) $(PhSe)_2$ (20 µM) + GSH (200 µM) + 1 min at 37 °C; (D) $(PhSe)_2$ (20 µM) + GSH (200 µM) + 1 min + H_2O_2 (200 µM) + 1 min at 37 °C. The controls assays with $(PhSe)_2$ (20 µM) + H_2O_2 (200 µM) and GSH (200 µM) + H_2O_2 (200 µM) not modified the spectra of $(PhSe)_2$ and GSH, respectively. Absorbance (Abs) was monitored by spectrophotometry (200 - 400 nm). For details, see materials and methods. The inset graph shows glutathione peroxidase activity of $(PhSe)_2$.

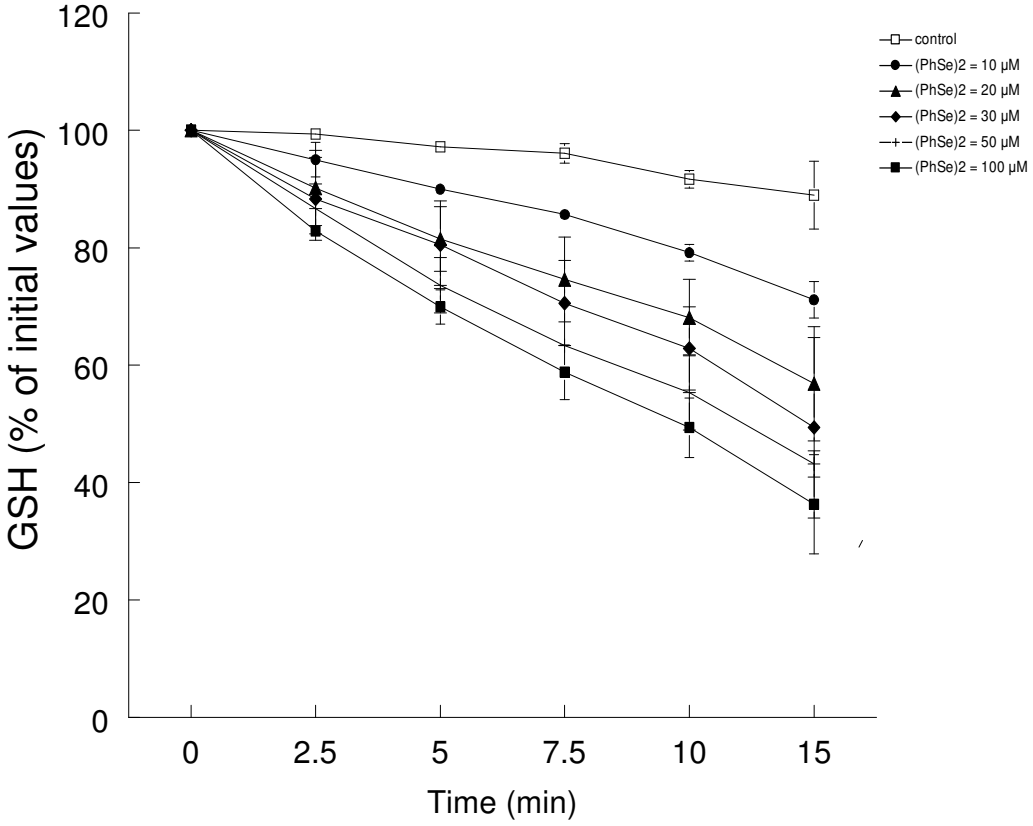

Figure 9 - Catalytic effects of $(PhSe)_2$ on glutathione oxidation in the presence of H_2O_2. The rate of glutathione oxidation was evaluated at different timepoints (0, 2.5, 5, 7.5, 10 and 15 min). Data are expressed as percent of reduced glutathione in the basal condition (time = 0; absence of diphenyl diselenide) and presented as mean ± standard error of mean (SEM) from at least three independent experiments.

Table 1 - Effects of $(PhSe)_2$ on Cu^{2+}-induced conjugated dienes formation in human LDL.

$(PhSe)_2$ (μM)	c (Cu^{2+}) = 1.6mM			c (Cu^{2+}) = 5 mM			c (Cu^{2+}) = 10mM		
	Lag time (min)	Oxidation rate (Abs 234nm/min)	Ox. maximun (ODmax)	Lag time (min)	Oxidation rate (Abs 234nm/min)	Ox. maximun (ODmax)	Lag time (min)	Oxidation rate (Abs 234nm/min)	Ox. maximun (OD max)
0	99.2 ± 1.16	0.002340 ± 0.000178	0.406 ± 0.011	35.5 ± 1.53	0.006197 ± 0.000105	0.592 ± 0.014	22.3 ± 1.15	0.011900 ± 0.000872	0.487 ± 0.013
1	120.4 ± 1.47 *	0.001657 ± 0.000087*	0.357 ± 0.009*	--	--	--	--	--	--
3	123.7 ± 0.78*	0.001183 ± 0.000043*	0.286 ± 0.01*	59.5 ± 1.44*	0.007217 ± 0.000268*	0.582 ± 0.007	25.2 ± 1.17	0.007910 ± 0.000165 *	0.511 ± 0.031
10	134.1 ± 1.11*	0.000542 ± 0.000035*	ND	85.1 ± 1.13*	0.006160 ± 0.000227	0.534 ± 0.021*	53.5 ± 0.83*	0.006723 ± 0.000118*	0.497 ± 0.017
15	--	--	--	87.5 ± 1.12*	0.005007 ± 0.000173*	0.509 ± 0.017*	66.4 ± 1.4*	0.006077 ± 0.000096*	0.468 ± 0.011
20	ND	ND	ND	--	--	--	--	--	--
40	ND	ND	ND	108.8 ± 0.6*	0.002070 ± 0.000091*	0.365 ± 0.011*	72.0 ± 1.53*	0.002207 ± 0.000159*	0.375 ± 0.009*

Quantitative parameters were derived from the oxidation curves of Figure 1 (left). Results are presented as mean ± standard error of mean (SEM) for at least three independent experiments. (*) Statistically different ($p < 0.05$) when compared to control group. Lag phase is the intercept of the slope tangent of the absorbance curve in propagation phase with the abscissa axis. Oxidation rates were obtained from the slopes of the absorbance curves during the propagation phase. ND: not determined within the experimental time-frame. OD max: Maximum optic density at 234 nm.

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3.2.2 Efeito do $(PhSe)_2$ e do ebselen na morte celular induzida pelo peroxinitrito.

3.2.2.1 Artigo 5

Protective effect of diphenyl diselenide against peroxynitrite-mediated endothelial cell death: a comparison with ebselen

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Protective effect of diphenyl diselenide against peroxynitrite-mediated endothelial cell death: a comparison with ebselen

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Abstract

Superoxide ($O_2^{\bullet -}$) and nitric oxide ($^{\bullet}NO$) are generated by blood vessels and can rapidly react to produce peroxynitrite ($ONOO^-$), a powerful oxidant that modifies lipoproteins making them more atherogenic. This study was designed to determine the effect of diphenyl diselenide ($(PhSe)_2$, a new synthetic organoselenium compound under investigation) in comparison with the well known ebselen, on peroxynitrite-mediated endothelial damage. Bovine aortic endothelial cells (BAEC) in primary cultures were treated with authentic peroxynitrite and cell viability, intracellular gluthatione (GSH) content and gluthatione peroxidase (GPx) activity were assessed. Experimental results showed that a long pre-incubation (24 h) with $(PhSe)_2$ (0.5 and 1μM) protected endothelial cells from the damage promoted by peroxynitrite exposure, in a more effective way than ebselen. The intracellular levels of GSH were almost completely consumed by peroxynitrite and although the compounds did not restore the normal levels, $(PhSe)_2$ *per se* increases significantly GSH in a concentration-dependent manner. This effect may be related with the significant increase in cellular GPx activity promoted by this compound, which revealed to be even more active as a glutathione peroxidase mimic, than ebselen. In conclusion, our data suggest a new role for $(PhSe)_2$ as a potential anti-atherogenic agent.

Key words: peroxynitrite, atherosclerose, diphenyl diselenide, endothelial cells, apotosis.

1 Introduction

Atherosclerosis and subsequent vascular diseases are the main cause of morbidity and mortality in the developed world. Vascular oxidative injury and LDL oxidation in the intima of arteries have been recognized as important steps in atherogenesis, leading to endothelial cell dysfunction associated with a low level inflammatory state (Chisolm and Steinberg, 2000; Lusis, 2000; Young and McEneny, 2001; Steinberg and Witztum, 2002). It has been reported that an overproduction of reactive oxygen and nitrogen species in the vascular endothelium occurs very early in the atherogenic process, suggesting a link between reactive oxidant species, LDL oxidation and endothelial cells dysfunction and apoptosis (Ohara et al., 1993; Alexander, 1995; Dimmeler and Zeiher, 2000).

Peroxynitrite ($ONOO^-$) represents a biologically relevant oxidizing and nitrating agent that is formed from the diffusion-limited reaction between nitric oxide ($^{\bullet}NO$) and superoxide ($O_2^{\bullet-}$). Of particular importance, peroxynitrite is a strongly reactive and short-lived species that can promote oxidative molecular damage (Koppenol, 1998). In addition to the generation of a pro-oxidant species, the formation of peroxynitrite results in decreased bioavailability of $^{\bullet}NO$, therefore diminishing both its salutary physiological functions (Moncada and Higgs, 1993; Radi et al., 2001) and its strong antioxidant actions over free radical and metal-mediated processes (Rubbo et al., 1994; Radi 1996). The ability of $ONOO^-$ to oxidize and covalently modify a wide range of biomolecules, including DNA, proteins and lipids, can affect important cellular functions in such a way that cell homeostasis may be compromised and subsequently apoptosis or necrosis may occur (Salgo et al., 1995; Ischiropoulos and al-Mehdim, 1995; Radi et al., 1991).

The involvement of $ONOO^-$ in atherosclerosis has been suggested either by its ability to oxidize LDL or by the extensive nitration of protein tyrosine, detected in human atherosclerotic lesions (Graham et al., 1993; Guy et al., 2001). Therefore, it is important to counteract $ONOO^-$ damaging effects. One of the possible strategies is to intercept this reactive species so that potentially sensitive biological targets can not be reached, preventing damage (Arteel et al., 1999). Organoselenium compounds may fulfill such requirements, in a similar way to ebselen that reacts with peroxinitrite efficiently. In fact, the second order rate constant for this reaction is one of the highest for a low-molecular-weight compound with peroxinitrite, as known so far

(Masumoto and Sies, 1996; Masumoto et al., 1996). Organoselenium compounds prevent protein from oxidation and nitration (Arteel et al., 1998; Briviba et al., 1996) and protect against DNA damage caused by $ONOO^-$ (Roussyn et al., 1996). Additionally, it has been reported that ebselen prevents the recruitment of leukocytes into inflamed tissues by attenuating $ONOO^-$-mediated IL-8 gene expression (Jozsef and Filep, 2003). On the other hand, two clinical studies showed that ebselen improves the neurological outcome of acute ischemia stroke and subarachnoid hemorrhage (Saito et al., 1998; Yamaguchi et al, 1998; Ogawa et al, 1999), pointing to the therapeutic significance of organoselenides.

Similarly to ebselen, diphenyl diselenide [$(PhSe)_2$], another organoselenium compound, has been demonstrated to have important antioxidant properties. In fact, this compound reacts very efficiently with hydroperoxides and organic peroxides, mimicking the glutathione peroxidase enzyme. Moreover, the gluthatione peroxidase-like activity of $(PhSe)_2$ has been shown to be even higher than that of ebselen, displaying also a lower toxicity (Nogueira et al., 2004). Of note, recently, some of us have demonstrated that $(PhSe)_2$ protects human platelets against lipoperoxidation induced by SNP, a NO donor, and leads to the recovering of GPx inactivation (Posser et al., 2006). Additionally, $(PhSe)_2$ has shown a protective role in a variety of experimental models associated with the overproduction of free radicals in brain and liver (Rossato et al., 2002; Meotti et al, 2004; Ghisleni et al.,2003; Burger et al., 2004; Borges et al., 2005).

Trying to contribute to the clarification of the potential protective role of $(PhSe)_2$ in atherogenesis, the aim of this study was to evaluate the protection afforded by this compound on peroxynitrite-induced endothelial cells death, by using primary cultures of bovine aortic endothelial cells (BAEC) as a model. In parallel, ebselen was used as a reference. Although cellular effects of ebselen have been widely described, no reports exist concerning $(PhSe)_2$. Our data indicate that $(PhSe)_2$ is able to decrease the programmed cell death in BAEC stimulated by bolus addition of peroxynitrite in a more efficient way then ebselen.

2 Materials and Methods

2.1 Materials

General laboratory chemicals and some specific ones, namely, collagenase, gelatine, streptomycin/penicillin, MTT, Hoechst 33258, ortho-phthaldeyde, GSH and glutathione reductase were obtained from Sigma Chemicals (St. Louis, MO). For cell culture, Dulbecco's modified Eagle's medium (DMEM), trypsin 0.25 %, fungizone, fetal bovine serum (FBS) and phosphate-buffered saline (PBS) pH 7.4, were purchased from Gibco-Invitrogen. $(PhSe)_2$ was synthesized as previously described (Paulmier et al., 1986). Analysis of the 1H NMR and 13C NMR spectra showed that the compound obtained presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of $(PhSe)_2$ (99.9%) was determined by GC/HPLC.

2.2 Primary cultures of bovine aortic endothelial cells

Bovine aortic endothelial cells (BAEC) were obtained from thoracic aorta by treatment with collagenase (2 mg/ml). Cells were cultivated on gelatine-coated tissue culture plastic in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml fungizone at 37 ºC, in a humidified atmosphere of 5 % CO_2. Endothelial cells were identified by their cobblestone morphology and uptake of fluorescently labelled acetylated LDL. Cells were subcultured at confluences and used between the fourth and the seventh passage. Prior to the experiments, cells at 80 % of confluence were starved in serum-free medium for 24 hours.

2.3 Peroxynitrite syntesis

Peroxynitrite was synthesized by using a quenched flow reactor, as previously described (Dinis et al., 2002). Briefly, an aqueous solution of 0.6 M $NaNO_2$ was mixed rapidly with an equal volume of 0.7 M H_2O_2 containing 0.6 M HCl and immediately quenched with the same volume of 1.5 M NaOH. The solution was then frozen at -20 ºC for approximately 24 h. At this temperature, peroxynitrite concentrates into a dark yellow top layer (freeze fractionation), which was removed and passed through a short column of MnO_2 for residual H_2O_2 remotion according to Uppu et al. (1996). Then it was stored at -80 ºC under N_2 atmosphere. Before use,

$ONOO^-$ was always quantified from the absorbance at 302 nm in 1 N NaOH (ε = 1670 $M^{-1}cm^{-1}$).

2.4 Peroxynitrite-induced oxidative stress

Peroxynitrite-induced oxidative stress in BAEC was performed as described by Brito et al., (2006). In brief, cells were washed and equilibrated with PBS (Phosphate buffered saline with calcium and magnesium) pH 7.4 for 5 min. Afterwards, cells were exposed to $ONOO^-$ (final concentration 500 µM), for 10 min and then they were washed and replaced in culture medium for 6 h. The same volumes of either 10 mM NaOH (vehicle control) or decomposed $ONOO^-$ ($ONOO^-$ was decomposed in PBS or 10 mM NaOH overnight) were used as controls. When required, cells were pre-incubated with $(PhSe)_2$ or ebselen in a medium free of fetal bovine serum for 24 h. At the end of the indicated time, the incubation medium was gently removed and the cells were incubated with peroxynitrite as described above. In these conditions, the compounds were not present in the incubation medium thoughout the experiment with peroxynitrite.

2.5 Cell Viability

Cells ($0.5x10^6$ cells/well) seeded in 6 well plates were incubated in a medium free of fetal bovine serum with various concentrations of $(PhSe)_2$ or ebselen for 24 h. Cell viability was then assessed by the MTT test according to Denizot and Lang (1986). Briefly, after the incubation period, the medium was removed, the cells were washed with PBS and further, 1 ml of PBS containing 100 µl of MTT (1g/L) was added to each well for 1 h at 37 ºC. Formazan crystals were dissolved in DMSO and the absorbance was read at 530 nm. Results were expressed as a percentage of control cells.

2.6 Nuclear morphology

Cells were fixed with freshly prepared 4 % (w/v) paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) for 15 min at room temperature and subsequently stained with Hoechst dye 33258 (5µg/ml) for 15 min, washed with PBS and mounted by using PBS/glycerol (3:1, v/v). Then, cells were examined by fluorescence microscopy, with a Nikon fluorescence microscope. Stained nuclei were scored according to the condensation and staining characteristics of chromatin. Apoptotic nuclei were identified by condensed chromatin and nuclear fragmentation

and were counted in at least five random microscopic fields (400 x) per sample. Only cells that remained attached to the plates were observed. Mean values were expressed as the percentage of apoptotic nuclei.

2.7 Measurement of cellular reduced and oxidized glutathione

Intracellular levels of GSH and GSSG were determined by a fluorimetric assay as previously described (Hissin and Hilf, 1976). In brief, cells were scrapped with 0.6 M perchloric acid and recovered into 100 mM sodium phosphate buffer containing 5 mM EDTA, pH 8.0 at 4 ºC. Samples were centrifuged at 14 000 rpm at 4 ºC for 10 min. A volume of 100 µl of supernatant was incubated with 100 µl of *ortho*-phthaldehyde (0.1% w/v in methanol) and 1.8 ml of 100 mM Na_2HPO_4 for 15 min at room temperature. For the GSSG determination, the supernatant was previously treated with N-ethylmaleimide (NEM), for 30 min and further incubated with *ortho*-phthaldehyde in 100 mM NaOH. Fluorescence intensity was read in a Perkin-Elmer LS 50B spectrofluorometer at an emission wavelength of 420 nm with an excitation wavelength of 350 nm (slits width 3.5 nm). Cellular GSH and GSSG contents were calculated by using concurrently run standard curves and expressed as nmoles of GSH or GSSG per milligram of cellular protein. Cellular protein content was quantified by the Bio-Rad protein assay dye, using bovine serum albumin as the standard.

2.8 Glutathione peroxidase activity

2.8.1 Measurement of cellular Glutathione peroxidase activity

Cellular gluthatione peroxidase (GPx) activity was measured as described before (Bem et al., 2006). Briefly, 40 µl of washed cells ($2x10^6$ cells), to remove any compound previously added, were placed in a 96 well plate and were incubated in 0,2 ml of a medium containing 50 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, 1 mM reduced glutathione (GSH), 1 U of glutathione reductase (GR) and 0.15 mM NADPH, at room temperature for 5 min. After addition of 10 µl of 4 mM H_2O_2, the rate of NADPH consumption was monitored at 340 nm, for 5 min in a Bio-Tek Synergy HT multi-detection microplate reader. The activity was followed by the decrease in NADPH absorption at 340 nm. Appropriate controls were carried out without cells and were subtracted. GPx activity was calculated from the NADPH

extinction coefficient value (6.22 mM^{-1} cm^{-1}) and expressed as nmol of NADPH consumed per minute.

2.8.2 Measurement of Glutathione peroxidase-like activity

The GPx like activity of diphenyl diselenide was evaluated by the method of Flohe and Gunzler (1984). The assays were performed at 37 °C in 2 ml of medium containing 50 mM potassium phosphate buffer, pH 7.0, 1 mM ethylene diamine tetraacetic acid (EDTA), 1 mM reduced glutathione (GSH), 1 U of glutathione reductase (GR) and 0.15 mM NADPH. Increasing concentrations of $(PhSe)_2$ were added to the mixture and the reaction was initiated by addition of H_2O_2 (0.2 mM final concentration). The reaction was carried out with gentle stirring, for 2 min, and the activity was followed by the decrease in NADPH absorption at 340 nm. Appropriate controls were carried out without $(PhSe)_2$ and were subtracted. In parallel, ebselen was used as a reference compound with glutathione peroxidase–like activity.

2.9 Statistical analysis

All data were expressed as mean ± SEM of at least three independent assays, each one in duplicate. Differences between groups were analysed by one-way analysis of variance (ANOVA) followed by Tukey multiple range test when appropriate. Values of $p < 0.05$ were considered statistically significant.

3 Results

3.1 Effect of diphenyl diselenide on cell viability

In order to evaluate the potential beneficial effects of diphenyl diselenide on peroxynitrite–mediated cell death, we started our experiments assessing the appropriate non-toxic concentration of diphenyl diselenide to be used in the study. Thus, a 24 h concentration-response study was conducted by exposing BAEC to concentrations of $(PhSe)_2$ ranging from 0.5 to 8 µM. Cell viability was evaluated by the MTT assay. As shown in Figure 1, there was no significant decrease in viability over a 24 h exposure to $(PhSe)_2$ concentrations below 2 µM. However, for higher concentrations a concentration-dependent decrease was observed. Similar results were observed with ebselen in a parallel assay. This compound was used in all the performed assays, as a reference organoselenium compound. On basis of Figure 1

results, the maximal non toxic concentration of $(PhSe)_2$ or ebselen used in the subsequent experiments was 1 μM.

3.2 Effect of diphenyl diselenide on peroxynitrite-mediated apoptotic changes

To investigate eventual citoprotective effects of $(PhSe)_2$ on the settings of peroxynitrite exposure, BAEC were pre-incubated with two different concentrations, 0.5 and 1 μM, of either $(PhSe)_2$ or ebselen for 24 h and then exposed to 500 μM of authentic peroxynitrite, as described in section 2. It should be highlighted that the compounds under study ($(PhSe)_2$ or ebselen) were not present during or after cells treatment with peroxynitrite. Previously, some of us have demonstrated that BAEC exposition to high ONOO- concentrations (500 and 600 μM) for 6 h induced apoptosis (Brito et al., 2006). Now, as shown in Figure 2, in the same assay conditions, 80% of the cells treated with $ONOO^-$ exhibited nuclear condensation, a hallmark of apoptosis, but cells pre-treated with $(PhSe)_2$ markedly reverted the programmed cell death. As evidenced in the same figure, $(PhSe)_2$ in a 0.5 μM concentration inhibited apoptosis by about 50% whereas ebselen at the same concentration only inhibited apoptotic cell death by about 20%.

To confirm the nature of cell death, the plasma membrane integrity, a feature of necrosis, was assessed by measuring LDH release to the medium. Such as previously demonstrated (Brito et al., 2006), LDH release from the cells exposed to $ONOO^-$ was not significantly different from the control cells. Also, LDH release from BAEC pre-incubated with either $(PhSe)_2$ or ebselen for 24 h, and then exposed to $ONOO^-$ was not significantly different from the control (data not shown), stressing the apoptotic cell death induced by peroxynitrite.

3.3 Effect of Diphenyl diselenide on intracellular glutathione

Since GSH efficiently reacts with peroxynitrite, protecting cells from its oxidative and nitrating-mediated damage (Klotz and Sies, 2003), and $(PhSe)_2$ efficiently protects cells from apoptotic death triggered by $ONOO^-$ (Fig 2), we investigated the mechanism underlying such protective effect of $(PhSe)_2$ by examining its influence on the cellular glutathione content. As shown in Figure 3A, a strong decrease in the intracellular GSH content and a concomitant increase of about two fold in the intracellular GSSG content were detected at the end of $ONOO^-$ treatment. Pre-incubation of BAEC with 0.5 μM $(PhSe)_2$ prevented some GSH

depletion mediated by $ONOO^-$, but no additional effect could be observed with 1 μM $(PhSe)_2$. On the other hand, of notice, $(PhSe)_2$ *per se* caused a significant increase in the GSH content in the absence of peroxynitrite. In fact, 0.5 μM $(PhSe)_2$ induced an increase in cellular GSH of about 1.6 fold relative to the control but this increase was not concentration dependent. Interestingly, although such increase in GSH, the GSSG content was remained constant in the absence of peroxynitrite, in contrast with that observed after cells exposition to $ONOO^-$ as referred above. Figure 3B show data from a parallel experiment with ebselen. Ebselen under our assay conditions neither prevented GSH depletion mediated by $ONOO^-$, nor per se, increased significantly the cellular GSH content.

3.4 Effect of Diphenyl diselenide on Glutathione peroxidase activity in BAEC

GSH and glutathione peroxidase (GPx) are critically involved in the cell protection against oxidative stress. GPx mediates the reduction of numerous reactive oxygen species, including peroxynitrite, at the expenses of GSH. On the other hand, it is well documented the glutathione peroxidase-like activity of organoselenium compounds, like ebselen. Therefore, we investigated the inducibility of GPx by $(PhSe)_2$ in BAEC. As shown in Figure 4, incubation of BAEC for 24 h with $(PhSe)_2$ led to a concentration-dependent increase in GPx activity. Actually, 0.5 and 1 μM $(PhSe)_2$ induced about 1.5 and 2 fold increase in GHS-Px activity, respectively. This effect was not so evident in ebselen treated cells (Fig 4). In fact, cells pre-treated with 1 μM of ebselen presented an induction of cellular GPx similar to that of cells pre-treated with 0.5 μM $(PhSe)_2$.

Additionally, we compared glutathione peroxidase-like activity of $(PhSe)_2$ and ebselen by using an *in vitro* assay as described in section 2. $(PhSe)_2$ presented a significant higher glutathione peroxidase-like activity than ebselen, for nearly all the concentrations tested, as evidenced in Figure 5.

Moreover, in the range of concentrations tested a plateau was reached at 30 μM ebselen, whereas for $(PhSe)_2$ a concentration-dependent GPx like activity was observed until 40 μM, the highest concentration used.

4 Discussion

Peroxynitrite in cells is a highly reactive specie, inducing a stress response. If the damage promoted by peroxynitrite exceeds the capacity of a target cell to restore a normal state, stress-responsive mechanisms lead to apoptosis induction (Brito et al., 1999; Lin et al., 1998; Virag et al., 1998). Although cellular effects of ebselen and its protective effect against peroxynitrite-induced damage has been already reported (Sies e Masumoto, 1997; Sies e Arteel, 2000; Klotz e Sies, 2003), no reports exist concerning $(PhSe)_2$ effect against such damage. In the present study, we examined the protection afforded by $(PhSe)_2$ against the damage induced by authentic peroxynitrite in BAEC, in comparison with ebselen.

The results presented here demonstrated, for the first time that pre-treatment of bovine aortic endothelial cells (BAEC) with low micromolar concentration of $(PhSe)_2$, significantly protected them from peroxynitrite-mediated injury (Fig 2). Moreover, it was evident that $(PhSe)_2$, at the lowest concentration used, was much more efficient than ebselen. On the other hand, our results suggest an apoptotic pathway for peroxynitrite-induced cell death, triggering condensation and fragmentation of nucleous without LDH leakage to the extracellular medium, according to those recently reported by Brito et al. (2006).

In recent studies using endothelial cells, ebselen demonstrated a potent inhibitory activity against either oxysterol-induced cytotoxicity (Wu and Huang, 2006), TNF-α-induced JNK activation, or adhesion molecule expression (Yoshizumi et al., 2004); also, it demonstrated a protective effect against H_2O_2-induced death in human umbilical vein endothelial cells (Ali et al., 2004). These results point to the potential utility of ebselen in preventing and/or treatment of endothelial cell disfunction, which has been suggested to be an early step in atherogenesis. However, no evidence has been yet reported concerning the effect of $(PhSe)_2$ on endothelial cells. Importantly, $(PhSe)_2$ display lower toxicity than ebselen; moreover, ebselen is a complex molecule and its synthesis is relatively expensive and time-consuming, in contrast with the synthesis of diselenides which is easier and cheaper.

The rate constant of peroxynitrite with GSH is not very high ($k_2 = 1.3x10^3 M^{-1} s^{-1}$; Koppenol et al., 1992) but the high intracellular concentration of this thiol (5–10 mM) makes GSH in a potent endogenous scavenger of peroxynitrite. In fact, peroxynitrite-mediated oxidations at cellular level increase when cells are depleted of

GSH and also high levels of GSH afford protection (Denicola and Radi, 2005). Accordingly, we observed a strong decrease in intracellular GSH content after peroxynitrite treatment. Pre-incubation with organoselenium compounds did not prevent GSH depletion. Of note, $(PhSe)_2$ *per se* stimulated a significant increase in the intralellular GSH pool (Fig 3), while ebselen cause only a slight increase in GSH content.

The antioxidant properties of the organoselenocompounds has attracted much attention because glutathione peroxidase system represents one the major lines of defense against hydrogen and lipid peroxides and peroxynitrite (Parnham et al., 1987; Sies and Masumoto, 1997). Ebselen and $(PhSe)_2$, have revealed strong antioxidant properties that may result from their GPx-like activities (Nogueira et al., 2004). According to this, our data indicate that thiol peroxidase activity of $(PhSe)_2$ is much higher than that of ebselen (Fig. 5), in agreement with the study of Wilson and collaborators (1989), which showed that $(PhSe)_2$ was about 1.6 times more effective than ebselen, as a gluthatione peroxidase-mimetic.

It is known that the selenoprotein gluthatione peroxidase (GPx) is able to reduce peroxynitrite efficiently, preventing both oxidation and nitration reactions (Sies et al., 1997). Thus, the reaction of GPx is considered a biologically efficient detoxication pathway *in vivo* (Arteel et al, 1999). Our results point to the thiol peroxidase-like activity of $(PhSe)_2$ as a plausible mechanism underlying its protective role against peroxynitrite-induced cell death, similarly to ebselen. In fat, GPx activity of BAEC was significantly increased in basal conditions after incubation with low micromolar concentration of $(PhSe)_2$, while ebselen caused a modest improvement in this activity (Fig 4). The decrease in intracellular GSH content after treatment with peroxynitrite, even in presence of organoselenium compounds, could be explained by the fact that the system of GPx plus GSH works catalytically in order to reduce peroxynitrite to nitrite at expenses of GSH (Sies et al., 1997).Then, we can propose that $(PhSe)_2$ has a key role in peroxynitrite detoxification, either directly by increasing the GSH content, a peroxynitrite scavenger and a substrate of GPx, or indirectly by improving the GPx activity in BAEC.

From the present findings, we conclude that $(PhSe)_2$, like ebselen, has a significant protective effect against peroxynitrite-mediated cell death. To our knowledge, our study is the first to show the effect of $(PhSe)_2$ on GPx improvement in bovine aortic endothelial cells, suggesting an intracellular mechanism underlying the

protection afforded by organoselenium compounds. It is noteworthy that these findings may shed new light on the pharmacological basis for the clinical applications of organoselenium compounds as a potential antiatherosclerotic compound.

Figures

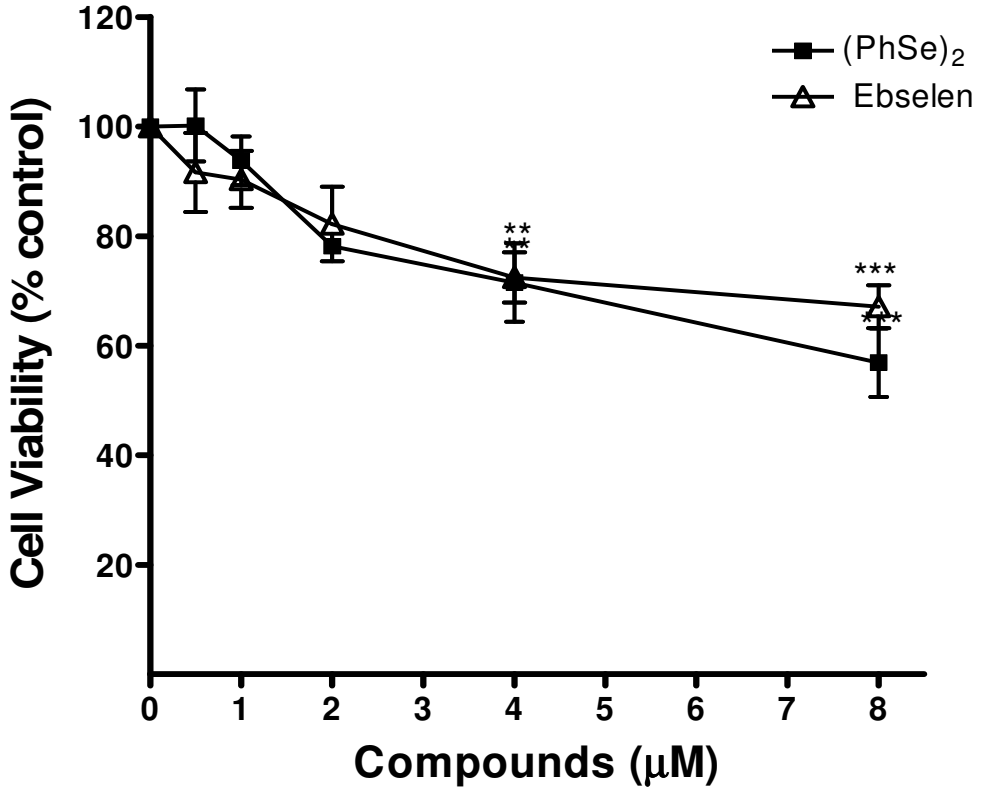


Figure 1. Effect of $(PhSe)_2$ on endothelial cell viability, as compared with ebselen. Confluent bovine aortic endothelial cells (BAEC) were incubated with different concentrations of $(PhSe)_2$ or Ebselen (0–8 μM) for 24 h, used as a reference compound. Cell viability was determined as described in Materials and Methods. Results were expressed as the % of the control i.e. the cells without added compounds. Values are mean ± SEM from at least three independent experiments, each one assayed in duplicate. ** $p < 0.01$ and *** $p < 0.001$ vs. control.

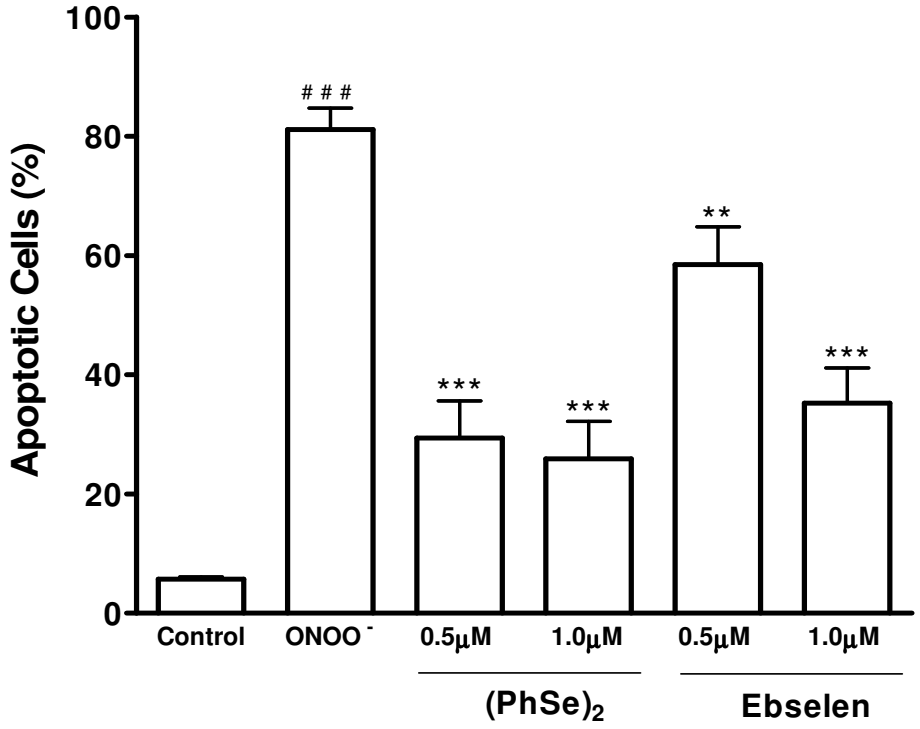
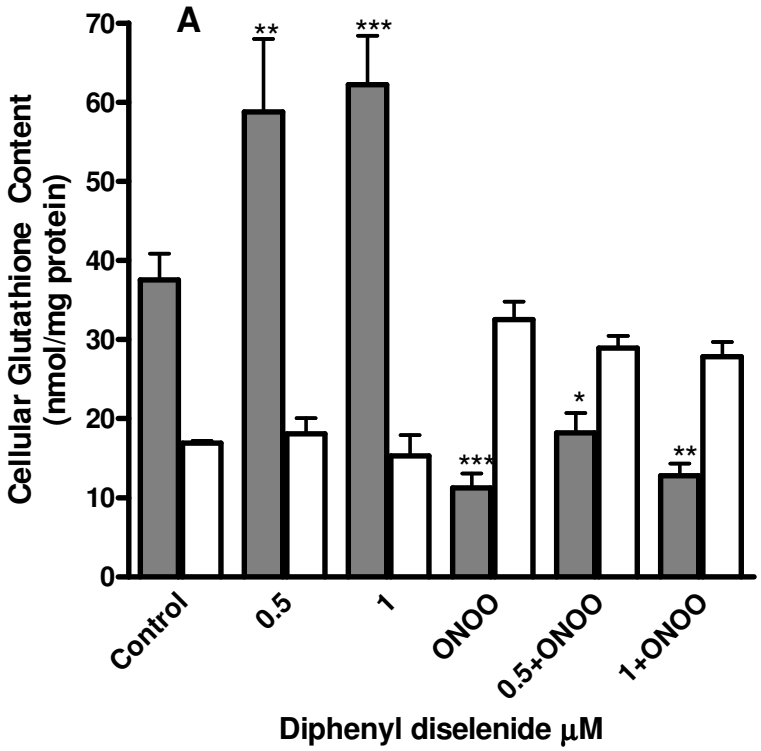


Figure 2. $(PhSe)_2$ prevents peroxynitrite-mediated apoptotic changes in endothelial cells, more efficiently than ebselen. Serum private confluent bovine aortic endothelial cells were pre-incubated with the indicated concentrations of $(PhSe)_2$ or ebselen for 24 h. Then, the cells were washed with PBS and treated with 500 μM of authentic peroxynitrite in PBS (1ml/well) for 10 min. Subsequently, PBS was removed and replaced by fresh serum-free media and incubated at 37 ℃ for 6 h. Morphological apoptotic changes were assessed by nuclear morphology observation under a fluorescent microscope after nuclei staining with Hoechst 33258, being considered apoptotic cells all those with condensed or fragmented nuclei. Results were expressed as the % of the total cell population. Control refers to the experiment in similar conditions without peroxynitrite and compounds. Data are mean ± SEM from at least three independent experiments each one assayed in duplicate. $^{###}$ $p < 0.001$ vs. control; ** $p < 0.01$ and *** $p < 0.001$ vs. peroxynitrite-treated cells.


A
Cellular Glutathione Content
(nmol/mg protein)
70
60
50
40
30
20
10
0
**
***
***
*
**
Control
0.5
1
ONOO
0.5+ONOO
1+ONOO
Diphenyl diselenide μM

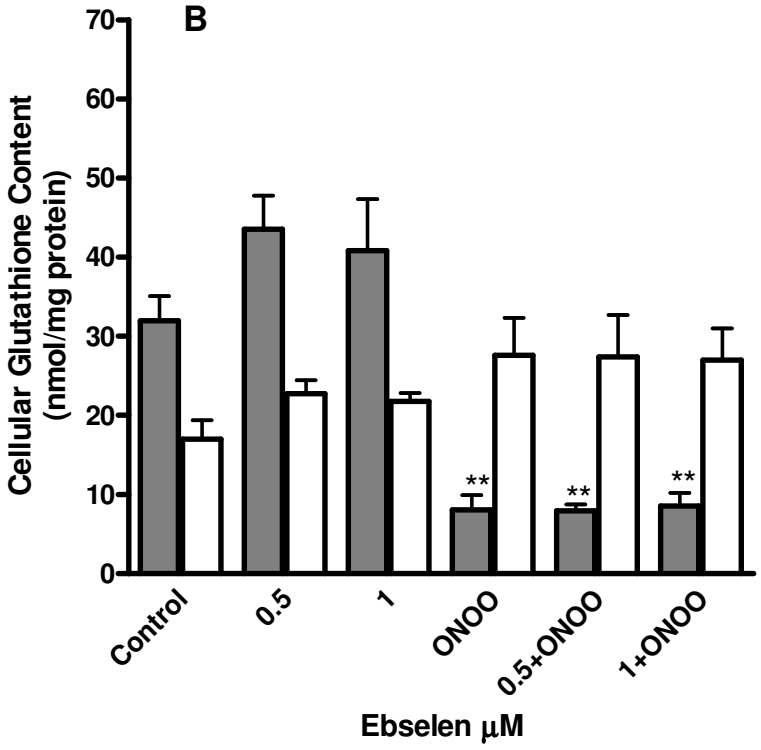

B
Cellular Glutathione Content
(nmol/mg protein)
70
60
50
40
30
20
10
0
**
**
**
Control
0.5
1
ONOO
0.5+ONOO
1+ONOO
Ebselen μM

Figure 3. (A) Effect of $(PhSe)_2$ on cellular reduced (grey bars) and oxidized (white bars) glutathione contents in the absence and presence of authentic peroxynitrite. Bovine aortic endothelial cells were pre-incubated with the indicated concentrations of $(PhSe)_2$ for 24 h. Subsequently, cells were washed with PBS and some of them were treated with 500 μM of peroxynitrite in PBS (1ml/well) for 10 min. GSH and GSSG were quantified immediately at the end of the peroxynitrite addition, as described in Materials and Methods. Control refers to incubated BAEC alone. Values are mean ± SEM of five experiments each one in duplicate. **(B)** The same experiment with ebselen, a selenocompound used as reference. ** $p < 0.01$; *** $p < 0.001$ vs control.

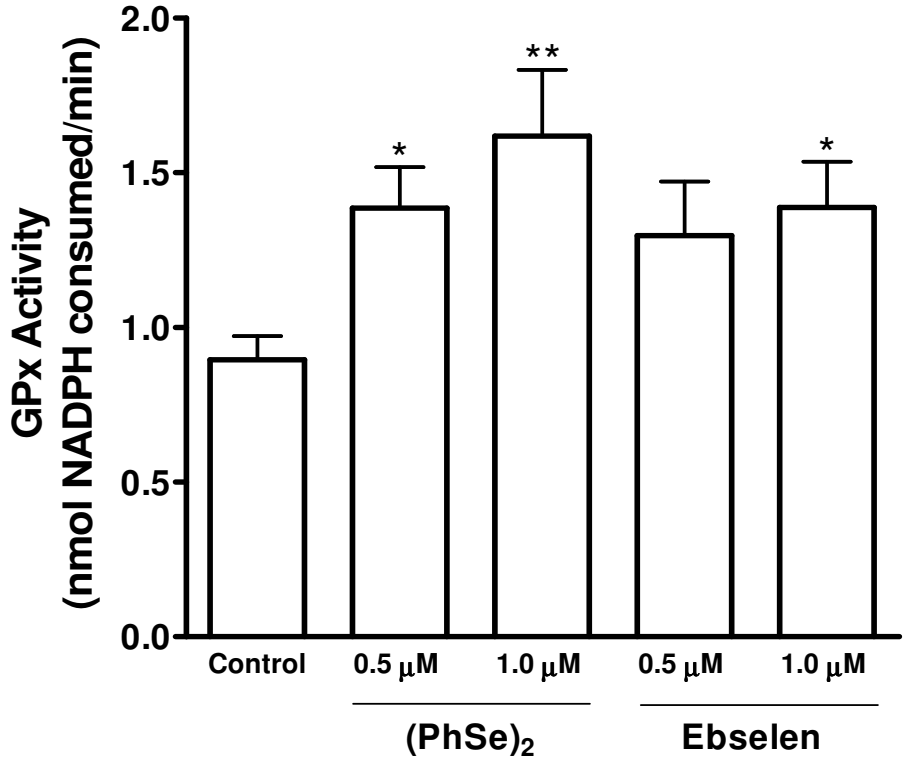


Figure 4. Effect of $(PhSe)_2$ on cellular GPx activity. Bovine aortic endothelial cells were pre-incubated with the indicated concentrations of $(PhSe)_2$ or ebselen for 24 h. Cellular GPx activity was measured as described in Materials and Methods and expressed as nmol NADPH consumed per minute. Ebselen was used for comparison. Values are mean ± SEM from at least three independent experiments each one assayed in duplicate.* $p < 0.05$; ** $p < 0.01$ vs control.

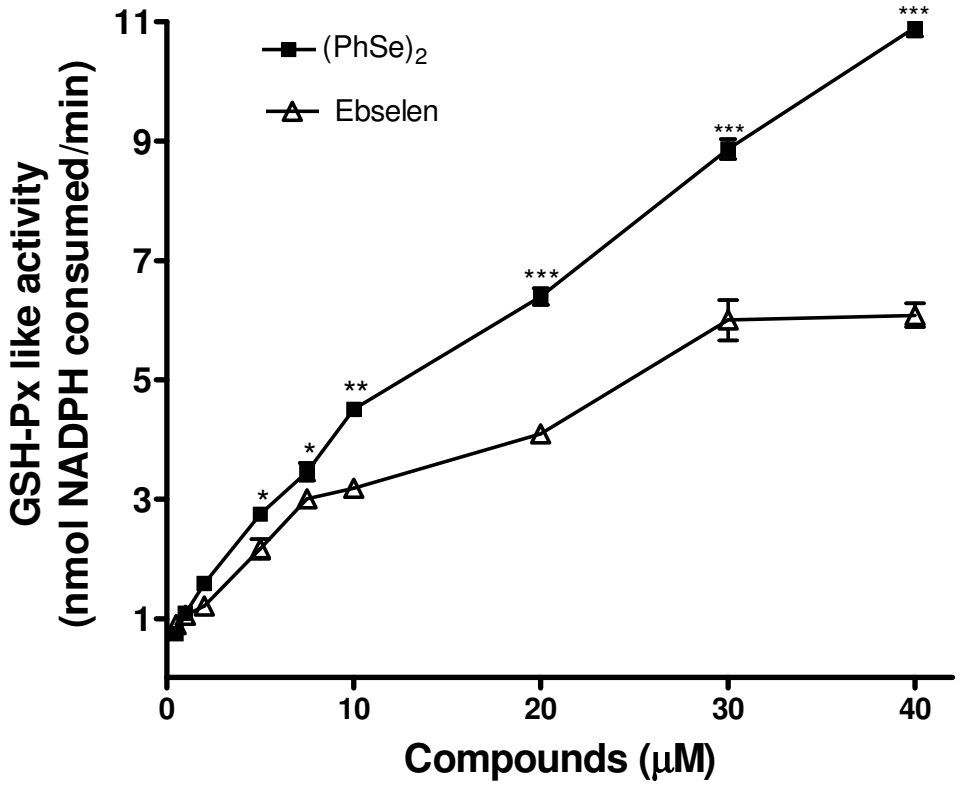


Figure 5. Glutathione peroxidase-like activity of $(PhSe)_2$, as compared with ebselen. GPx activity was measured as described in Materials and Methods and expressed as nmol NADPH consumed per minute. Ebselen was used as a glutathione peroxidase mimic. Data are mean ± SEM of five experiments each one in duplicate. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs the same ebselen concentration.

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4 DISCUSSÃO

O interesse por estudos químicos e bioquímicos dos compostos orgânicos de selênio (Se) tem aumentado consideravelmente nas últimas três décadas principalmente devido ao fato de que vários destes compostos apresentam importante atividade antioxidante (Andersson et al., 1994; Arteel e Sies, 2001), a qual possivelmente está relacionada à capacidade destes em mimetizar a atividade da enzima glutationa peroxidase (GPx) (Weldel,et al., 1984; Muller et al, 1984).

O primeiro exemplo desta classe de compostos foi o ebselen, extensivamente estudado na última década. Este composto de baixa toxicidade (Fischer et al., 1988) demonstrou propriedades antiinflamatória, antiaterosclerótica e citoprotetora em modelos experimentais *in vivo* e *in vitro* (Schewe, 1995; Takasago et al.,1997; Porciuncula et al., 2003; Brodsky et al., 2004). Além disso, tem sido empregado com sucesso em estudos clínicos no tratamento de pacientes com aneurisma hemorrágico (Saito el al., 1998) e isquemia aguda (Yamaguchi et al., 1998).

O disseleneto de difenila ($(PhSe)_2$) compartilha importantes características químicas e farmacológicas com o ebselen. De fato, o $(PhSe)_2$ demonstrou ser mais ativo como mimético da GPx que o ebselen (Wilson et al., 1989). Diversos trabalhos têm demonstrado que o $(PhSe)_2$ possui outras propriedades farmacológicas, tais como efeitos anti-úlcera (Savegnago et al., 2006), antiinflamatório e antinociceptivo (Nogueira et al., 2003a; Zasso et al., 2005), hepatoprotetor (Borges et al., 2005; 2006), entre outras. Além disso, este organocalcogênio demonstrou papel protetor em uma variedade de modelos experiementais associados com a super produção de radicais livres (Ghisleni et al., 2003; Burger et al., 2004; Posser et al., 2006).

Recentes dados evidenciaram que o $(PhSe)_2$ não foi tóxico quando administrado agudamente em ratos e camundongos em doses que demonstraram efeitos anti-inflamatório e anti-nociceptivo (Nogueira et al., 2003a; Zasso et al., 2005), todavia a exposição a doses muito altas causou sinais de toxicidade em roedores (Maciel et al., 2000; Jaques-Silva et al., 2001). Baseando-se nestas evidências e pelo fato de que não existem dados na literatura que avaliem o efeito do $(PhSe)_2$ em não roedores, um dos objetivos desta pesquisa foi avaliar o efeito da suplementação oral com $(PhSe)_2$ em coelhos. Os resultados obtidos nos artigos 1 e 2 demonstraram que a suplementação oral com $(PhSe)_2$ por oito meses foi relativamente segura, mesmo na maior dose utilizada neste protocolo de estudo (30

ppm), a qual está muito acima dos valores nutricionais preconizados para a ingestão de Se em humanos.

Os dados apresentados nos artigos 1 e 2 referem-se aos parâmetros bioquímicos e marcadores de estresse oxidativo avaliados no sangue, fígado, rim e cérebro de coelhos suplementados com $(PhSe)_2$. A ingestão de diferentes doses de $(PhSe)_2$ (0.3; 3 e 30 ppm) não modificou as atividades da enzimas hepáticas ALT e AST, bem como os níveis sanguíneos dos marcadores de lesão renal, uréia e creatinina ao longo do tratamento. Estes resultados diferem da hepatotoxicidade observada em roedores após a exposição com altas doses de $(PhSe)_2$ (Maciel et al., 2000; Jaques-Silva et al., 2001; Meotti et al., 2003). Desta forma, pode-se inferir que a toxicidade do $(PhSe)_2$ depende da via de administração bem como da espécie animal considerada.

O $(PhSe)_2$ tem sido descrito como um potencial agente antioxidante (Nogueira et al., 2004). Neste sentido foram avaliados vários parâmetros bioquímicos relacionados ao estresse oxidativo. Dentre as enzimas antioxidantes analizadas, foi observado somente um aumento isolado da catalase (CAT) nos eritrócitos dos coelhos expostos a 3 ppm de $(PhSe)_2$. Em relação aos antioxidantes não enzimáticos, os níveis de Vitamina C foram consistentemente reduzidos no sangue, fígado e cérebro dos coelhos expostos a uma dose relativamente alta de $(PhSe)_2$, aspecto que indica uma possível toxicidade do composto. Estes resultados contrastam com os obtidos com camundongos expostos por 10 dias a altas doses de $(PhSe)_2$, no qual um aumento do conteúdo hepático de vitamina C foi observado (Jaques-Silva et al., 2001). Todavia, os níveis de TBARS, um importante marcador do estresse oxidativo, não foram alterados no sangue e nos tecidos dos animais após a exposição com o $(PhSe)_2$, indicando que o composto não induziu a peroxidação lipídica.

A atividade da δ-ALA-D e os níveis hepáticos de tióis não protéicos (SHNP) diminuíram em roedores após exposição crônica com $(PhSe)_2$ (Maciel et al., 2000; Jaques-Silva et al., 2001). Em contraste com estes resultados, verificou-se um aumento na atividade da δ-ALA-D no sangue, fígado e cérebro, bem como nos níveis hepáticos de SHNP nos coelhos. O aumento da atividade da δ-ALA-D no tecido hepático pode estar associado ao aumeuto nos níveis de SHNP neste tecido, já que

grupamentos sulfidrila estão presentes no sítio ativo da enzima (Barnard et al., 1977).

O consumo de 30 ppm de $(PhSe)_2$ aumentou significativamente a deposição de selênio no tecido adiposo peri-renal sem causar alterações nos níveis de Se no soro. Estes resultados indicam que devido a sua alta lipossolubilidade, o $(PhSe)_2$ é rapidamente redistribuído do sangue para os tecidos ricos em lipídeos.

Na segunda etapa dos estudos *in vivo,* apresentados no Artigo 3 desta tese, investigaram-se os efeitos hipocolesterolêmico e antioxidante do $(PhSe)_2$ em coelhos submetidos a uma dieta rica em colesterol. De fato, o estresse oxidativo é um dos fatores que relaciona a hipercolesterolemia com a aterosclerose (Harrison et al., 2003). Neste sentido, inúmeros estudos demonstraram que a adição de antioxidantes a uma dieta rica em colesterol reduziu significativamente a severidade de lesões ateroscleróticas (Wojcicki, et al., 1991, Negis et al, 2006; Jenner et al, 2007).

Os resultados apresentados no Artigo 3 demonstraram efeitos benéficos da suplementação com $(PhSe)_2$ em não roedores expostos a uma dieta hipercolesterolêmica. A ingestão de uma dieta rica em colesterol induziu a um expressivo aumento nos níveis de colesterol total e dos marcadores de estresse oxidativo (TBARS e espécies reativas) no sangue dos coelhos. Por outro lado, a suplementação oral com $(PhSe)_2$ reduziu significativamente os níveis de colesterol e reestabeleceu os níveis dos marcadores de estresse oxidativo aos níveis do controle. Da mesma forma, o consumo de uma dieta rica em colesterol aumentou a susceptibilidade à lipoperoxidação induzida por Cu^{2+} no fígado dos coelhos, porém deve-se destacar que o consumo concomitante de $(PhSe)_2$ pelos animais foi capaz de prevenir a lipoperoxidação induzida. Estes efeitos antioxidantes do $(PhSe)_2$ estão de acordo com estudos anteriores que demonstraram que o $(PhSe)_2$ foi um efetivo agente antioxidante, prevenindo a produção de TBARS induzida por diferentes agentes pro-oxidantes (Rossato et al., 2002; Posser et al., 2006; Puntel et al., 2007).

De maneira semelhante aos resultados apresentados no artigo 2, a atividade da δ-ALA-D hepática e cerebral, bem como os níveis hepáticos de SHNP aumentaram com a suplementação oral com $(PhSe)_2$ e este comportamento foi mantido mesmo com o consumo concomitante de colesterol pelos animais. Cabe ressaltar que o período de exposição ao $(PhSe)_2$ foi mais curto (45 dias) e que a dose do referido composto foi o equivalente a 10 ppm, estando portanto, entre a

média e a maior dose utilizada nos ensaios de toxicidade crônica (Artigos 1 e 2). Em relação aos níveis de vitamina C, observamos a mesma situação demonstrada no ensaio de toxicidade, ou seja, a suplementação com $(PhSe)_2$ provocou a diminuição dos níveis hepáticos de vitamina C.

Em relação aos três artigos que abrangem os estudos *in vivo* apresentados nesta tese, pode-se inferir que a administração oral de $(PhSe)_2$ produziu baixos indícios de toxicidade em não roedores. Neste ínterim, cabe ressaltar que a diminuição dos níveis de vitamina C no sangue e no fígado dos animais que receberam a dose mais alta do composto configuraram o único sinal de toxicidade após exposição prolongada com $(PhSe)_2$. Ademais, os resultados sugerem uma associação entre as mudanças ocorridas nos parâmetros bioquímicos relacionadas ao estresse oxidativo no sangue, fígado e cérebro devido a hipercolesterolemia e os efeitos benéficos do $(PhSe)_2$, melhorando o status oxidativo e reduzindo os níveis de colesterol em coelhos hipercolesterolêmicos.

Considerando que o estresse oxidativo está diretamente envolvido no desenvolvimento da aterosclerose através do dano oxidativo às lipoproteínas, principalmente à lipoproteína de baixa densidade (LDL), investigou-se o efeito do $(PhSe)_2$ *in vitro* na proteção contra a oxidação de LDL humana isolada. Os resultados apresentados no Artigo 4 demonstraram que o $(PhSe)_2$ protegeu as LDLs contra oxidação induzida por Cu^{2+} e pelo AAPH, um gerador de radical hidroxila, de forma concentração dependente.

Como citado anteriormente, acredita-se que o principal mecanismo pelo qual o $(PhSe)_2$ exerce suas atividades antioxidantes está relacionado à sua atividade mimética a GPx (Nogueira et al., 2004). Do ponto de vista molecular e mecanístico, torna-se importante salientar que o $(PhSe)_2$ preveniu a peroxidação do soro humano induzida por Cu^{2+} e que este efeito foi acompanhado pelo consumo dos grupamentos sulfidrila da amostra, demonstrando desta forma a atividade tiol peroxidase deste composto. De acordo com esta hipótese, o $(PhSe)_2$ foi capaz de reduzir o peróxido de hidrogênio às custas de glutationa reduzida (GSH). Esta idéia foi reforçada pelos estudos espectroscópicos que demonstraram a interação entre o $(PhSe)_2$ e a glutationa reduzida, resultando na formação de um intermediário químico que teve sua estabilidade afetada pela presença do peróxido de hidrogênio.

Um efeito interessante deste estudo (Artigo 4) foi o fato de o $(PhSe)_2$ mostrar-se eficiente como inibidor da oxidação da LDL em momentos distintos do processo

oxidativo, prevenindo a formação de dienos conjugados mesmo quando a lipoproteína já estava parcialmente oxidada. Estes resultados são interessantes porque demonstraram que a atividade tiol peroxidase do composto é importante também na prevenção da geração de produtos secundários da lipoperoxidação.

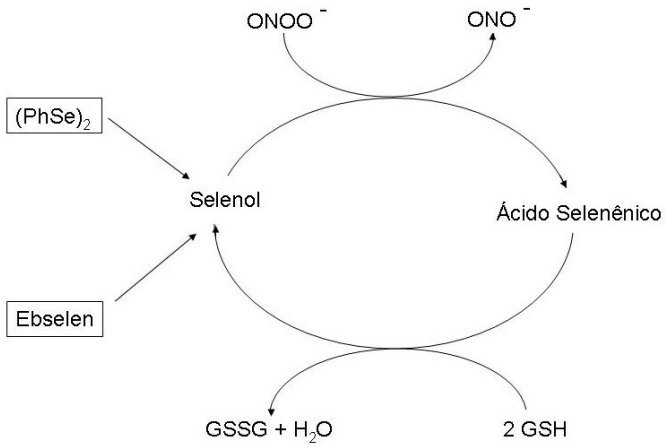
Outro aspecto muito importante deste estudo foi evidenciado pela capacidade do $(PhSe)_2$ em prevenir a perda da fluorecência do triptofano induzida pela oxidação das LDLs pelo Cu^{2+}. Este resultado indica que além dos efeitos benéficos relacionados à oxidação dos componentes lipídicos da LDL, este organocalcogênio também previne a oxidação das moléculas protéicas da LDL, nomiadamente a Apo B100, contribuindo com um mecanismo adicional na inibição do processo aterogênico.

Na segunda parte dos estudos *in vitro* apresentados nesta tese (Artigo 5) avaliou-se o efeito protetor do $(PhSe)_2$ em comparação ao ebselen, na morte celular induzida por peroxinitrito ($ONOO^-$) em cultura primária de células endoteliais de aorta bovina (BAEC). Inúmeros trabalhos sugerem o papel protetor do ebselen contra os efeitos nocivos do peroxinitrito (Sies e Masumoto, 1997; Sies e Arteel, 2000; Klotz e Sies, 2003), indicando a sua utilidade na prevenção e/ou tratamento da disfunção endotelial, um dos processos iniciadores da aterosclerose (Yoshizumi et al., 2004; Ali et al., 2004; Wu e Huang, 2006). Todavia, nenhuma evidência foi reportada até o momento sobre o efeito do $(PhSe)_2$ nas células endoteliais e a sua relação com o peroxinitrito.

O peroxinitrito tem a capacidade de oxidar e modificar covalentemente uma grande variedade de biomoléculas incluindo DNA, proteínas e lipídeos, afetando importantes funções celulares que comprometem a homeostase induzindo à morte celular (Salgo et al., 1995; Ischiropoulos e al-Mehdim, 1995).

Os resultados apresentados no último artigo desta tese (Artigo 5) demonstraram pela primeira vez que o $(PhSe)_2$ foi capaz de diminuir a morte celular programada nas BAECs, induzida pela adição em bolus de peroxinitrito, de maneira mais eficiente que o ebselen. Arteel e colaboradores (1999) demonstraram que o ebselen é capaz de reduzir eficientemente o peroxinitrito a nitrito através do intermediário selenol ás custas de grupamentos tióis redutores. Similar a este mecanismo, este trabalho propõe que selenol resultante da reação do $(PhSe)_2$ com grupamentos tióis, (Mugesh e Singh, 2000; Nogueira et al. 2004) notavelmente GSH, reduz peroxinitrito a nitrito. O ácido selenenico, produzido nesta reação é

subsequentemente reduzido pela GSH estabelecendo um ciclo catalítico capaz de proteger as células dos efeitos nocivos do peroxinitrito (Esquema 1).



Esquema 1: Mecanismo catalítico proposto para o Ebselen e $(PhSe)_2$ na redução do peroxinitrito a nitrito (Adaptado de Arteel et al., 1999).

A glutationa (GSH) é um importante *scavenger* de peroxinitrito (Koppenol et al., 1992), sendo que existe uma relação inversa entre o aporte de GSH e as oxidações mediadas pelo peroxinitrito (Denicola e Radi, 2005). De fato, observou-se uma expressiva depleção no conteúdo intracelular de GSH após o tratamento com peroxinitrito. O $(PhSe)_2$ *per se* estimulou um aumento significativo no conteúdo intracelular de GSH, todavia nenhum dos organocalcogênios preveniu a depleção provocada pelo peroxinitrito.

A selenoproteína glutationa peroxidase (GPx) é capaz de reduzir eficientemente o peroxinitrito prevenindo ambas reações de oxidação e nitração (Sies et al., 1997). Os dados do artigo 5 indicaram que a atividade tiol peroxidase do $(PhSe)_2$ foi muito superior à do ebselen, fato este que está de acordo com o estudo de Wilson e colaboradores (1989), os quais demonstraram que o $(PhSe)_2$ foi cerca de 1,6 vezes mais efetivo como mimético da GPx que o ebselen. Estes resultados sugerem a atividade tiol peroxidase dos organocalcogênios como um dos possíveis mecanismo de seu efeito protetor contra os efeitos nocivos do peroxinitrito.

De fato, a atividade basal da GPx nas BAECs aumentou significativamente após a incubação com o $(PhSe)_2$, enquanto o ebselen induziu a um modesto aumento na atividade celular desta enzima. A diminuição no conteúdo intracelular de GSH após o tratamento com o peroxinitrito, mesmo na pesença dos

organocalcogênios, pode ser explicada pelo fato que o sistema GSH/GPx trabalha cataliticamente no sentido de reduzir o peroxinitrito O, a nitrato, às custa de GSH (Sies et al., 1997). Desta forma, o Artigo 5 propõe que o $(PhSe)_2$ apresenta um papel chave na detoxicação do peroxinitrito, tanto diretamente por aumentar o conteúdo intracelular de GSH, *scavenger* de peroxinitrito e substrato para a GPx, ou indiretamente aumentando a atividade da GPx nas BAECs.

Em relação aos estudos *in vitro* apresentados nesta tese, observamos importantes efeitos benéficos do $(PhSe)_2$ tanto nos ensaios utilizando LDL humana isolada, onde o organocalcogênio inibiu a peroxidação lipídica e a oxidação protéica das LDLs, como no modelo de células endoteliais (BAECs), em que preveniu a morte celular induzida pelo peroxinitrito. Em ambos os casos, a partir das evidências observadas no decorrer deste estudo, sugere-se que o efeito protetor do $(PhSe)_2$ está relacionado à sua atividade tiol peroxidase.

Finalmente, os resultados obtidos neste estudo contribuem para um melhor entendimento das bases toxicológicas e farmacológicas da aplicabilidade clínica do $(PhSe)_2$. Neste contexto pode-se inferir que o $(PhSe)_2$ é um composto com baixa toxicidade em não roedores e com atrativas propriedades farmacológicas relacionada à atividade antiaterogênica.

5 CONCLUSÕES

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

- ✓ A suplementação oral prolongada com $(PhSe)_2$ mostrou-se relativamente segura para os coelhos. O único aspecto de toxicidade foi evidenciado pela diminuição dos níveis sanguíneos, hepáticos e cerebrais de vitamina C na maior dose testada. Os parâmetros relacionados à toxicidade hepática e renal estiverem dentro da normalidade em todo o período do tratamento.
- ✓ A suplementação oral com $(PhSe)_2$ reduziu os níveis de colesterol bem como o estresse oxidativo em coelhos hipercolesterolêmicos.
- ✓ O $(PhSe)_2$ inibiu a peroxidação lipídica e a oxidação da apoproteína de LDL humana isolada de maneira concentração dependente, sendo que este fenômeno parece estar relacionado com a sua atividade tiol peroxidase.
- ✓ O $(PhSe)_2$ foi capaz de diminuir a morte celular programada nas BAECs estimulada pela adição em bolus de peroxinitrito, de maneira mais eficiente que o Ebselen.

6 PERSPECTIVAS

Tendo em vista os promissores resultados obtidos nesta tese, poderíamos aprofundar ainda mais os estudos relacionados aos aspectos toxicológicos, a potencial ação antiaterogênica e a capacidade em neutralizar o peroxinitrito do $(PhSe)_2$, considerando o mecanismo de ação para estes efeitos. Dessa forma, poderíamos realizar este estudo a partir da concretização dos seguintes objetivos:

- ✓ Investigar os possíveis efeitos toxicológicos do $(PhSe)_2$ em não roedores após exposição aguda pelas vias intra-peritoneal e sub cutânea.
- ✓ Avaliar a capacidade do $(PhSe)_2$ em reduzir a progressão da aterosclerose experimental e melhorar o relaxamento vascular em coelhos alimentados com colesterol.
- ✓ Avaliar a habilidade do $(PhSe)_2$, em previnir as modificações oxidativas nas porções lipídicas e protéicas das LDLs, mediadas por oxidantes de relevância fisiológica como a ferrilmioglobina e o peroxinitrito.
- ✓ Investigar os mecanismos intracelulares envolvidos na proteção mediada pelo $(PhSe)_2$ contra as ações do peroxinitrito em células endoteliais.
- ✓ Investigar a habilidade do $(PhSe)_2$ em reduzir a peroxidação lipídica mediada pelo peroxinitrito em tecidos biológicos.

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8 ANEXO

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