UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS NATURAIS E EXATAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA TOXICOLÓGICA

MECANISMOS ENVOLVIDOS NO EFEITO ANTIOXIDANTE DO DISSELENETO DE DIFENILA NO DANO OXIDATIVO CAUSADO PELA EXPOSIÇÃO À FUMAÇA DO CIGARRO EM RATOS JOVENS

TESE DE DOUTORADO

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Santa Maria, RS, Brasil
2009

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por

Cristiane Luchese

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

Orientadora: Prof[a] Dr[a] Cristina Wayne Nogueira

Santa Maria, RS, Brasil
2009

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

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

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

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Santa Maria, 09 de setembro de 2009

“Que estranha é a sina que cabe a nós mortais! Cada um de nós está aqui para uma temporada; com que propósito não se sabe [...] Os ideais que tem iluminado meu caminho, e repetidamente me tem renovado a coragem para enfrentar a vida com ânimo, são a Bondade, a Beleza e a Verdade [...] O único homem que está isento de erros é aquele que não arrisca acertar”.

Albert Einstein

AGRADECIMENTOS

Aos meus pais, por estarem sempre presentes, pelo exemplo de responsabilidade, amor e dedicação. A vocês que sempre fizeram todo o esforço para me possibilitar à melhor educação, o melhor caráter, os melhores valores. À minha irmã, Lisiane, pela ajuda, pela amizade, pelas conversas, pelos conselhos, e por ter feito o legítimo papel de irmã. Ao meu anjo da guarda, Franciane (*in memoriam*) por ter feito parte da minha vida em um dos momentos mais importantes. Saiba que você, apesar de todas as limitações, me ensinou muito e faz muita falta, mas estará para sempre no meu coração!

Ao Guilherme, meu amor, por existir, pela compreensão, pela amizade, pelo amor incondicional dedicado durante todos esses anos e pelo apoio. Meu amor, obrigada por entender os dias que eu chegava cansada, que chegava cheirando a laboratório (mas especificadamente, a rato) e que tinha que ficar estudando. Você é muito especial na minha vida!

À professora Cristina, minha orientadora, que não poupou esforços para que eu chegasse até aqui, sempre me incentivando, tentando me passar seus conhecimentos; extremamente prestativa e disponível. Cris, obrigada por tudo: pela oportunidade, pelos ensinamentos, pelo tempo dedicado, pelas sugestões, por ter me possibilitado uma formação intelectual sólida, e principalmente por ter acreditado em mim quando eu mais precisei. Você é um exemplo de profissional que vou levar para o resto da vida, principalmente pela sua integridade moral e honestidade no trabalho acadêmico.

Ao GZ, pelo incentivo, pela confiança, pela amizade, pelo exemplo de dedicação, pelos momentos de descontração que foram muito importantes e inesquecíveis durante todos esses anos no laboratório.

À Marina, obrigado pela amizade, pelo companheirismo, pelo ombro amigo, pelos conselhos, pelas parcerias, pela confiança. Você foi muito especial nesta trajetória. Te adoro Loka!!

À Eluza, minha querida amiga, você é um exemplo de profissional, pela sua coragem e pela sua dedicação. Obrigado Lú por ter me ajudado, me aconselhado, me ensinado, pelas parcerias, pelas idéias, pela amizade e companheirismo.

As minhas queridas ICs, que hoje, com muito orgulho, já são mestrandas: Bibi, Aninha e Si. Cada uma de vocês foi muito importante nesta etapa. Obrigada por tudo! Eu adoro vocês e vou levá-las no meu coração para o resto da vida!

Obrigada aos demais amigos do laboratório e do laboratório do professor GZ pela convivência, pelas conversas, conselhos, ensinamentos, risadas, piadas, e desculpa pela poluição sonora.

Aos antigos colegas de laboratório, em especial a Fran, a Lu, ao Schumacher e ao Ricardo por toda a ajuda.

Aos colegas de outros laboratórios, em especial aos laboratórios do professor João, do professor Félix e do professor Oscar.

Aos professores do Programa de Pós-graduação em Bioquímica Toxicológica.

À CAPES, pelo auxílio financeiro durante a realização deste trabalho.

À Universidade Federal de Santa Maria e ao Programa de Pós-Graduação em Bioquímica Toxicológica, pela oportunidade de realização deste curso.

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria

MECANISMOS ENVOLVIDOS NO EFEITO ANTIOXIDANTE DO DISSELENETO DE DIFENILA NO DANO OXIDATIVO CAUSADO PELA EXPOSIÇÃO À FUMAÇA DO CIGARRO EM RATOS JOVENS

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Data e Local da Defesa: Santa Maria, 2009

A fumaça do cigarro é uma mistura complexa de diversos constituintes identificados, entre eles, substâncias reativas (espécies reativas de oxigênio e nitrogênio), as quais podem estar relacionadas com o desenvolvimento de várias doenças em adultos e crianças. Os pulmões e cérebro estão entre os órgãos mais afetados pela exposição à fumaça do cigarro. Entretanto, as crianças são mais suscetíveis aos danos causados pela exposição passiva à fumaça do cigarro do que os adultos. Para proteger os tecidos do dano oxidativo causado por esta exposição são utilizados antioxidantes. O disseleneto de difenila [$(PhSe)_2$] é um composto orgânico de selênio que apresenta diversos efeitos farmacológicos descritos, entre eles, o antioxidante. Entretanto, o mecanismo pelo qual este composto exerce seus efeitos antioxidantes ainda não foi elucidado. Portanto, o presente trabalho visa estudar os efeitos da exposição passiva à fumaça do cigarro nos pulmões e no cérebro de ratos jovens, em dois protocolos experimentais de estresse oxidativo, e verificar o papel protetor do $(PhSe)_2$ nestes protocolos. Além disso, investigaram-se os mecanismos envolvidos no efeito antioxidante desse composto. Para isso, foram utilizados ratos jovens que foram submetidos a dois protocolos experimentais de estresse oxidativo. Em um primeiro protocolo experimental (P1) verificou-se o efeito da exposição passiva à fumaça de um, dois e três cigarros nas primeira, segunda e terceira semanas de vida, respectivamente. Em um segundo protocolo experimental (P2) foi verificado o efeito da exposição passiva à fumaça de quatro, cinco e seis cigarros nas primeira, segunda e terceira semanas de vida, respectivamente. Todos os animais em ambos os protocolos experimentais foram expostos à fumaça do cigarro diariamente, 15 minutos cada exposição, por um período de 20 dias. O animais que foram tratados com o $(PhSe)_2$, receberam diariamente pela via oral a dose de 0,5 mg/kg, imediatamente antes de cada exposição. No 21° dia, os animais foram eutanasiados e os pulmões e cérebro foram retirados para a análise da peroxidação lipídica (níveis de espécies reativas ao ácido tiobarbitúrico – TBARS), das defesas antioxidantes enzimáticas (atividade das enzimas superóxido dismutase (SOD), catalase (CAT), glutationa redutase (GR), glutationa peroxidase (GPx) e glutationa S-transferase (GST)), das defesas antioxidantes não-enzimáticas (níveis de tióis não-protéicos (SHNP) e ácido ascórbico) e da atividade da δ-aminolevulinato desidratase (δ-ALA-D). Os animais foram pesados diariamente, antes de cada exposição. O peso dos animais não alterou após a exposição à fumaça do cigarro em nenhum grupo experimental, em nenhum dos protocolos. Nos P1 e P2, os animais que foram expostos à fumaça do cigarro apresentaram um aumento da peroxidação lipídica no pulmão. Entretanto no cérebro, o aumento no TBARS foi verificado apenas no P2. Em relação às defesas antioxidantes não-enzimáticas, foi observado uma redução nos níveis dos parâmetros estudados nos animais expostos ao P1 e P2. Enquanto no cérebro, a exposição ao P1 aumentou os níveis de ácido ascórbico e a exposição ao P2 reduziu os níveis de SHNP e de ácido ascórbico. Quanto às defesas antioxidantes enzimáticas,

elas alteraram no pulmão apenas no P2. No cérebro, a atividade da CAT reduziu após a exposição ao P1, e a exposição ao P2 reduziu a atividade da CAT e da SOD. A atividade da δ-ALA-D foi alterada no P1 no pulmão e no P2 no cérebro. O tratamento com $(PhSe)_2$ restaurou os danos causados pela exposição passiva à fumaça do cigarro nos pulmões e cérebro dos ratos jovens. Além disso, os níveis de SHNP, o conteúdo de ácido ascórbico e a atividade da GST apresentaram um aumento *per se* nos pulmões dos animais tratados com $(PhSe)_2$. Para estudar o mecanismo pelo qual o $(PhSe)_2$ apresentou efeito antioxidante, verificou-se o efeito mimético *in vitro* deste composto orgânico de selênio (1 - 50µM) na atividade das enzimas dehidroascobato (DHA) redutase e GST. O $(PhSe)_2$ a partir da concentração de 5µM apresentou efeito mimético da atividade destas enzimas. Além disso, estudou-se o efeito do $(PhSe)_2$ (10 - 50µM) como *scavenger* dos radicais 1,1-difenil-2-picril-hidrazil ($DPPH^{\bullet}$) e 2,2'-azino-bis(3-etilbenztiazolina-6-ácido sulfônico) ($ABTS^{\bullet+}$), e na proteção da auto-oxidação do Fe^{2+}. Entretanto, o composto não apresentou efeito *scavenger*, nem protegeu da auto-oxidação do Fe^{2+}, descartando que esses mecanismos estariam envolvidos na ação antioxidante do composto. O envolvimento na síntese da glutationa (GSH) também foi estudado na tentativa de explicar os mecanismos pelos quais o $(PhSe)_2$ apresenta efeito antioxidante. Para isso, foi utilizado a butionina sulfoximina (BSO), uma substância que inibe a γ-glutamilcisteína sintetase, uma enzima envolvida na síntese da GSH. Verificou-se que o BSO bloqueou o efeito protetor do $(PhSe)_2$ na redução dos níveis de SHNP provocada pela exposição passiva à fumaça do cigarro no pulmão e fígado de ratos jovens. Com o presente trabalho conclui-se que o $(PhSe)_2$ apresenta efeito antioxidante por diferentes mecanismos. A ação do $(PhSe)_2$ em aumentar os níveis de SHNP, ácido ascórbico e a atividade da GST é um dos mecanismos do efeito antioxidante deste composto. O $(PhSe)_2$ apresentou atividade DHA redutase e GST-*like*, demonstrando um novo mecanismo do efeito antioxidante do composto. Além disso, a síntese da GSH está envolvida no efeito antioxidante do $(PhSe)_2$, uma vez que, bloqueando a síntese da GSH, ocorre um bloqueio no efeito antioxidante do composto.

Palavras-chave: Disseleneto de difenila, mecanismos antioxidantes, fumaça do cigarro, selênio, glutationa, ácido ascórbico.

ABSTRACT

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

ON THE MECHANISMS OF DIPHENYL DISELENIDE ANTIOXIDANT EFFECT ON THE OXIDATIVE DAMAGE CAUSED BY CIGARETTE SMOKE EXPOSURE TO RAT PUPS

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Date and Place of the defense: Santa Maria, 2009

Cigarette smoking is a complex mixture of many constituents identified, among them reactive substances (reactive oxygen and nitrogen species), which are capable of initiating or promoting oxidative damage. Lungs and brain are affected by cigarette smoke exposure. Exposure to cigarette smoke is related to development of diseases in children and adults. However, children are more susceptible than adults to damage caused by cigarette smoke. Thus, the use of antioxidants is a good alternative to protect tissues of oxidative damage caused by cigarette smoke exposure. Diphenyl diselenide [$(PhSe)_2$] is an organoselenium compound that presents pharmacological effects, among them the antioxidant effect. Nevertheless, the mechanism involved in antioxidant effect of $(PhSe)_2$ was not been elucidated. Therefore, this study was performed to study the effects of cigarette smoke passive exposure in lungs and brain of rat pups in two experimental protocols of oxidative stress. Moreover, the antioxidant effect of $(PhSe)_2$ in these experimental protocols was studied. Besides, the mechanisms involved in the antioxidant effect of $(PhSe)_2$ were investigated. Rat pups that were exposed to two experimental protocols were used. In a first experimental protocol (P1), the effect of exposure to one, two and three cigarettes during the first, second and third weeks of live, respectively, was studied. In a second experimental protocol (P2), the effect of exposure to four, five and six cigarettes during the first, second and third weeks of life, respectively, was carried out. The duration of each exposure was 15 min. Animals were exposed to cigarette smoke during 20 days (3 weeks). Immediately before each exposure, animals that were treated with $(PhSe)_2$ received daily an oral dose of 0.5 mg/kg. At the end of the experimental exposure period (3 weeks), rat pups were euthanized, and lungs and brain were removed for analyses of lipid peroxidation, enzymatic antioxidant defenses (superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), glutathione S-transferase (GST) activities) and non-enzymatic defenses (non-protein thiols (NPSH) and ascorbic acid levels). Rat pups were daily weighed, before each exposure. The weight of animals was not changed after cigarette smoke exposure neither to P1 nor to P2. In both experimental protocols, animals that were exposed to cigarette smoke showed an increase in lipid peroxidation in lungs. In brain, an increase in thiobarbituric acid reactive species (TBARS) was observed only in P2. Levels of non-enzymatic antioxidant defenses decreased in lungs of animals exposed to P1 and P2. In brain, exposure to P1 increased ascorbic acid levels and exposure to P2 reduced NPSH and ascorbic acid levels. Enzymatic antioxidant defenses changed in P2 in lungs of rat pups. In brain, the activity of CAT was reduced after exposure to P1, while SOD and CAT activities were decreased after exposure to P2. Treatment with $(PhSe)_2$ restored the oxidative damage caused by cigarette smoke exposure in lungs and brain of rat pups. Moreover, NPSH levels, ascorbic acid content and GST activity showed an increase *per se* in lungs of animals treated with $(PhSe)_2$. The mechanisms involved in antioxidant effect of $(PhSe)_2$ (1 - 50 µM) were studied. To this end,

dehydroascorbate (DHA) reductase and GST activities were determined. $(PhSe)_2$ at concentration of 5 µM demonstrated DHA reductase and GST-like activities. Furthermore, the scavenger effect of 2,2'-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radicals, and the protection of Fe^{2+} autooxidation were studied. However, the compound had no scavenger effect or protected Fe^{2+} autooxidation, discarding that these mechanisms are involved in the antioxidant effect of $(PhSe)_2$. Synthesis of glutathione (GSH) was also studied as a possible mechanism involved in antioxidant effect of $(PhSe)_2$. For this, buthionine sulfoximine (BSO), a substance that inhibits γ-glutamilcystheine synthase activity, an enzyme involved in the synthesis of GSH was used. BSO blocked the protective effect of $(PhSe)_2$ in reducing NPSH levels caused by cigarette smoke passive exposure in lungs and liver of rat pups. In this study it was concluded that $(PhSe)_2$ had antioxidant effect by different mechanisms. The action of $(PhSe)_2$ to increase NPSH and ascorbic acid levels, and GST activity is one of mechanisms of the antioxidant effect of this compound. $(PhSe)_2$ presented DHA reductase and GST-like activities, demonstrating a new mechanism of antioxidant effect of the compound. Furthermore, the synthesis of GSH is involved in the antioxidant role of $(PhSe)_2$, since blocking the synthesis of GSH, presented the antioxidant effect of this compound.

Keywords: Diphenyl diselenide, antioxidant mechanisms, cigarette smoke exposure, selenium, glutathione, ascorbic acid.

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LISTA DE ABREVIATURAS

δ-ALA-D - δ-Aminolevulinato desidratase

ABTS - 2,2'-Azino-bis(3-etilbenztiazolina-6-ácido sulfônico)

BSO - Butionina sulfoximina

CAT – Catalase

CDNB - 1-Cloro-2,4-dinitrobenzeno

DHA – Dehidroascorbato

DPPH - 1,1-Difenil-2-picril-hidrazil

$(PhSe)_2$ - Disseleneto de difenila

ERNs - Espécies reativas de nitrogênio

EROs - Espécies reativas de oxigênio

GSSG - Glutationa oxidada

GPx - Glutationa peroxidase

GR - Glutationa redutase

GSH - Glutationa reduzida

GST - Glutationa S-transferase

SMSL - Síndrome da morte súbita do lactente

SOD - Superóxido dismutase

SHNP - Tióis não-protéicos

GLUT - Transportadores de glicose

SVCT - Transportadores saturáveis dependentes de sódio

SUMÁRIO

1. INTRODUÇÃO

A fumaça do cigarro é considerada um dos principais fatores de risco no desenvolvimento de doenças pulmonares, doenças cardio e cerebrovasculares, câncer, entre outras (Eiserich et al., 1995). A exposição passiva à fumaça do cigarro está relacionada com o desenvolvimento de doenças, tanto em adultos quanto em crianças (Gidding et al., 1994; Taylor et al., 1992). Entretanto, comparado com os adultos, as crianças são mais suscetíveis aos danos causados pela fumaça do cigarro, porque seus túbulos brônquicos são menores e seu sistema imune é menos desenvolvido (WHO, 1999; Tutka et al., 2002). Durante a lactação, a exposição passiva à fumaça do cigarro pode aumentar o risco de doenças e síndromes infantis como asma e bronquites (Klonoff-Cohen et al., 1995).

A fumaça do cigarro é uma mistura complexa de aproximadamente 5000 constituintes identificados (Green e Rodgman, 1996; Perfetti et al.,1998; Baker e Proctor, 2001). Entre os componentes presentes na fumaça do cigarro estão incluídas numerosas substâncias reativas, como uma grande variedade de aldeídos (Park, 1998), e espécies reativas de oxigênio (EROs) e nitrogênio (ERNs) (Pryor e Stone, 1993).

As espécies reativas presentes na fumaça do cigarro induzem o estresse oxidativo, promovendo um aumento na peroxidação lipídica e conseqüentemente uma perturbação dos sistemas de defesas antioxidantes no sangue e nos tecidos dos fumantes (Pryor e Stone, 1993). O dano oxidativo pode ser causado pela ação direta de substâncias reativas contidas na fumaça do cigarro, e também devido a eventos secundários (Tao et al., 2003; Ardite et al., 2006). O pulmão é o principal órgão que está em contato direto com os componentes da fumaça do cigarro. Este órgão apresenta defesas antioxidantes que o protegem contra os danos causados por espécies reativas (Foronjy e D'Armiento, 2006). O cérebro também é um órgão afetado pelos componentes presentes na fumaça do cigarro e este órgão é extremamente vulnerável ao estresse oxidativo. Assim como o pulmão, o cérebro também apresenta defesas antioxidantes que o protegem do estresse oxidativo causado pela exposição à fumaça do cigarro. Entretanto, o cérebro tem menos defesas antioxidantes quando comparado com o pulmão (Power et al., 2008).

As defesas antioxidantes não enzimáticas, como as vitaminas C, E, a glutationa reduzida (GSH), e as defesas antioxidantes enzimáticas, como a superóxido dismutase (SOD), a catalase (CAT), a glutationa peroxidase (GPx), a glutationa redutase (GR) e a glutationa S-transferase (GST), protegem os tecidos do dano oxidativo causado pelas EROs (Halliwell e

Gutteridge, 2000). Estudos têm mostrado que os antioxidantes, tanto endógenos quanto exógenos, apresentam importantes funções na prevenção de injúrias pulmonares causadas pela exposição a poluentes ambientais (Cross et al., 2002; Ho, 2002) e possuem um papel protetor na maioria das doenças causadas pela fumaça do cigarro (Tiwari, 2004).

Entretanto, quando o sistema de defesa antioxidante endógeno é insuficiente para conter o estresse oxidativo causado por espécies reativas, é necessário a utilização de antioxidantes exógenos. Neste sentido, diversos estudos têm demonstrado que moléculas contendo selênio são melhores antioxidantes (e, conseqüentemente melhores nucleófilos) do que antioxidantes clássicos, como por exemplo, as vitaminas e os flavonóides (Arteel e Sies, 2001). Por este motivo, tem aumentado o interesse no desenvolvimento de compostos orgânicos de selênio sintéticos, como, por exemplo, o disseleneto de difenila $(PhSe)_2$. O $(PhSe)_2$ é um composto orgânico de selênio que reduz a peroxidação lipídica induzida por uma variedade de oxidantes (Rossato et al., 2002; Nogueira et al. 2004). Além disso, o $(PhSe)_2$ apresenta diversas propriedades farmacológicas (Nogueira et al., 2003; Borges et al., 2005; Barbosa et al., 2006; Savegnago et al., 2006, 2007) destacando-se o potencial antioxidante evidenciado em modelos experimentais que envolvem o estresse oxidativo (Meotti et al., 2004; Santos et al., 2004, 2005a,b; Borges et al., 2008; Luchese et al., 2009; Prigol et al., 2009a). O mecanismo pelo qual o $(PhSe)_2$ apresenta atividade farmacológica é atribuído a ação antioxidante do composto.

Entretanto, os mecanismos pelos quais o $(PhSe)_2$ apresenta o efeito antioxidante não foram completamente elucidados. Sabe-se que a atividade antioxidante de diversos compostos orgânicos de selênio, tais como o ebselen e o $(PhSe)_2$, está relacionada, ao menos em parte, a suas atividades de mimetizar a enzima GPx (Nogueira et al., 2004; Zhao et al., 2004). Evidências na literatura indicam que o $(PhSe)_2$ apresenta atividade tiol peroxidase (Wilson et al., 1989; Meotti et al., 2004) e apontam o papel antioxidante do composto por modular algumas defesas antioxidantes não-enzimáticas, tais como os níveis de tióis não-protéicos e de ácido ascórbico (Barbosa et al., 2006, 2008; Borges et al., 2008; Luchese et al., 2009).

Considerando que o $(PhSe)_2$ apresenta propriedade antioxidante e que os mecanismos envolvidos na ação do composto não foram completamente elucidados, o presente trabalho estudou o efeito protetor do $(PhSe)_2$ sobre o dano oxidativo causado pela exposição passiva à fumaça do cigarro nos pulmões e cérebro de ratos jovens, bem como os possíveis mecanismos envolvidos na ação antioxidante deste composto orgânico de selênio.

2. REVISÃO BIBLIOGRÁFICA

2.1. Fumaça do cigarro

2.1.1. Componentes da fumaça do cigarro

A fumaça do cigarro é uma mistura complexa de aproximadamente 5000 constituintes identificados (Green e Rodgman, 1996; Perfetti et al., 1998; Baker e Proctor, 2001). Nos últimos 30-40 anos, diversos estudos foram realizados na tentativa de identificar a composição química da fumaça do cigarro, tanto quantitativamente quanto qualitativamente (Pryor et al., 1983; Jeffery, 1999). Entre os componentes presentes na fumaça do cigarro estão incluídas numerosas substâncias reativas, como uma grande variedade de aldeídos (Park et al., 1998), EROs e ERNs (Pryor e Stone, 1993), e diversos metais (WHO, 1992). Além disso, foram identificados compostos como os alcalóides de piridina entre eles, a nicotina, a amônia, a acroleína, os fenóis, o acetaldeído, as N-nitrosaminas; como os hidrocarbonetos aromáticos policíclicos como a benzopirina; como os gases de combustão,tais como o monóxido de carbono, os óxidos de nitrogênio, o cianeto de hidrogênio, os elementos traço; como os elementos radioativos α-emissores entre eles, o polônio, o rádio e o tório (Bogden et al., 1981; Jeffery, 1999). A proporção dos principais componentes em cada cigarro pode alterar-se dependendo da marca (Tabela 1).

A fumaça do cigarro pode ser dividida em duas fases: a fase particulada (alcatrão) e a fase gasosa (gás). Na fase particulada estão presentes hidrocarbonetos aromáticos policíclicos, nicotina, fenóis, cresol, β-naftilamina, N-nitrosonornicotina, benzopireno, elementos traço (níquel, arsênico, polônio), indol, catecol, entre outros (Pasupathi et al., 2009). Na fase gasosa estão presentes monóxido de carbono, ácido hidrociânico, acetaldeído, acroleína, amônia, formaldeído, óxidos de nitrogênio, nitrosaminas, hidrazina, entre outros (Pasupathi et al., 2009).

As espécies reativas e os radicais livres estão presentes nas duas fases da fumaça do cigarro. Ambas as fases são ricas em radicais livres contendo carbono, nitrogênio e oxigênio, assim como oxidantes não-radicais. A partir da análise de cada fase, foi estimado que um único *puff* contém aproximadamente 10^{14} radicais livres na fase particulada e 10^{18} radicais na

fase gasosa (Pryor et al., 1983). Além disso, estão incluídos vários compostos os quais são capazes de causar um aumento na geração de várias EROs tais como radical superóxido, peróxido de hidrogênio, peroxil e hidroperoxil. Estas EROs são capazes de iniciar ou promover um dano oxidativo causando uma peroxidação lipídica (Koul et al., 2001).

Na fase gasosa então presentes radicais orgânicos e inorgânico tais como, EROs, peróxidos, óxido nítrico, peroxinitrito e outros radicais livres e espécies reativas (Church e Pryor, 1985; Pryor e Stone, 1993). Tem sido demonstrado que na fase gasosa da fumaça do cigarro estão presentes radicais dos tipos alquil, alcoxil e peroxil (Pryor e Stone, 1993). O radical de óxido nítrico está presente na fumaça do cigarro em concentrações de 500-1000 ppm, representando uma das maiores fontes de radical de óxido nítrico exógeno ao qual os seres humanos são expostos. O radical de óxido nítrico reage rapidamente como o radical ânion superóxido formando o peroxinitrito e também reage com os radicais peroxil orgânicos (conhecidos por estarem presentes na fumaça do cigarro) formando alquil peroxinitritos (Padmaja e Huie, 1993). Na fase particulada, os radicais são estáveis e são predominantemente orgânicos tais como, a semiquinona, a qual está presente na matriz do alcatrão e pode reagir com o oxigênio formando o radical ânion superóxido. Nesta fase há também outras EROs, tais como o radical hidroxil e o peróxido de hidrogênio (Janoff et al., 1987).

Ambas as fases da fumaça do cigarro são nocivas e contêm grandes concentrações de substâncias tóxicas e compostos cancerígenos (IARC, 1992). Essas duas fases estão associadas com desenvolvimento de diversas doenças pulmonares, incluindo o câncer. A ativação metabólica, os mecanismos de desintoxicação e a toxicidade destes compostos têm sido amplamente estudados (Hecht, 1991). Embora seja bem estabelecido que a fase particulada contém um grande número de agentes cancerígenos (Smith et al., 2000), outras publicações sugerem que os produtos químicos na fase gasosa da fumaça do cigarro são de grande importância nos efeitos citotóxicos e cancerígenos em células epiteliais broncopulmonares (Witschi et al., 1985; Pouli et al., 2003). Após décadas de investigações, tornou-se evidente que não existe um único mecanismo envolvido na toxicidade da fumaça do cigarro.

Tabela 1 – Proporção em mg/cigarro dos principais componentes presentes no cigarro comercial da marca Hollywood Original KS®:

Constituintes	Concentração média
Alcatrão	9,0
Nicotina	0,7
Monóxido de Carbono	9,7
Hidrocarbonetos Polícíclicos Aromáticos	
- Benzopireno	0,000009
Compostos Carbonilados	
- Formaldeído	0,046700
- Acetaldeído	0,390360
- Acetona	0,207680
- Acroleína	0,073260
Fenóis	
- Fenol	0,011230
- m-cresol	0,002530
- p-cresol	0,005130
- o-cresol	0,003200
Amônia	0,007490
Ácido Cianídrico	0,121280
Bases Semi-Voláteis	
- Piridina	0,010550
pH	5,89
Misturas Orgânicas	
- Acrilonitrila	0,017340
- Benzeno	0,049260
- Tolueno	0,076980
- Estireno	0,008160
Aminas Aromáticas	
- 1-aminonaftaleno	0,000019
- 2-aminonaftaleno	0,000009
NO_X	0,287670

Fonte: Empresa Souza Cruz

2.1.2. Exposição à fumaça do cigarro na infância

A exposição à fumaça do cigarro pré- e pós-natal causa efeitos na mortalidade e na morbidade das crianças (Hofhuis et al., 2003). A exposição passiva à fumaça do cigarro pós-natal causa efeitos mais agudos à saúde das crianças (Hofhuis et al., 2003).

A exposição passiva à fumaça do cigarro durante a infância é um importante fator de risco para a síndrome da morte súbita do lactente (SMSL) (Anderson e Cook, 1997). Nessa síndrome ocorre a morte súbita e inesperada durante o sono, e normalmente afeta crianças com menos de um ano de idade, em que a história clínica, o exame físico, a necropsia e o exame do local do óbito não demonstram a causa específica do mesmo (Guntheroth e Spiers, 1992). Além disso, crianças que morreram de SMSL apresentaram maior concentração de nicotina no tecido pulmonar quando comparada com casos de morte onde não ocorreram SMSL, demonstrando que a exposição passiva à fumaça do cigarro é um fator para o desenvolvimento da SMSL (Anderson e Cook, 1997; McMartin et al., 2002). As possíveis razões para a associação entre a exposição passiva à fumaça do cigarro e a SMSL são anormalidades no desenvolvimento cerebral, com uma tendência à apnéia central (Milerad e Sundell, 1993) e distúrbios no mecanismo de controle respiratório (Anderson e Cook, 1997), incluindo uma reduzida resposta ventilatória à hipóxia (Hafstrom et al., 2000). Outras explicações plausíveis são um desenvolvimento pulmonar anormal nos lactentes (Tager et al., 1995) e a promoção de infecções respiratórias (Strachan e Cook, 1997).

Trabalhos têm demonstrado que crianças expostas à fumaça do cigarro durante o período gestacional e durante o período pós-natal apresentaram uma redução na função pulmonar (Stick et al., 1996; Dezateux e Stocks, 1997; Cook et al., 1998). Testes em recém-nascidos de mães fumantes demonstraram uma redução no fluxo expiratório forçado quando comparados com recém-nascidos de mães não-fumantes (Hanrahan et al., 1998; Hoo et al., 1998; Dezateux et al., 1999).

Estudos epidemiológicos demonstram que a exposição à fumaça do cigarro pré-natal e pós-natal pode causar alterações neurodesenvolvimentais e comportamentais em crianças, assim como uma capacidade intelectual reduzida, déficit de atenção e hiperatividade (Eskenazi e Castorina, 1999). Além disso, existe uma relação entre a exposição pré-natal de crianças e a incidência de detenções penais e de hospitalizações psiquiátricas por abuso de substância em meninos e meninas (Brennan et al., 2002).

As infecções meningocócicas também são incidentes em crianças expostas à fumaça do cigarro durante os períodos pré- e pós-natal (Kriz et al., 2000). Além disso, o risco de asma (Strachan e Cook, 1998a), reações alérgicas (Strachan e Cook, 1998b), tosse (Strachan e Cook, 1997) e chiado (Strachan e Cook, 1997) são sintomas relacionados com a exposição passiva à fumaça do cigarro em crianças lactentes.

Alguns estudos na literatura têm demonstrado o efeito da exposição passiva à fumaça do cigarro em ratos jovens (Anand e Anand, 1997; Florek et al., 1999; Subramaniam et al., 1999; Xia et al., 2009). O desenvolvimento das estruturas cerebrais e pulmonares de ratos começa durante o período gestacional e termina no período pós-natal. As três primeiras semanas de vida dos ratos é o período no qual as estruturas pulmonares e cerebrais estão em processo de desenvolvimento. Estas estruturas completam o desenvolvimento em torno de 21 dias após o nascimento dos animais (Yokoyama, 1983; Burri, 1974, 2006; Morgane et al., 2002, Tschanz et al., 2003). O desenvolvimento das estruturas nos ratos difere do desenvolvimento nos humanos. Os humanos durante o nascimento estão com as estruturas cerebrais formadas enquanto os ratos terão a formação completa 21 dias após o nascimento (Morgane et al., 2002). O fato das estruturas cerebrais e pulmonares completarem o desenvolvimento em torno de 21 dias após o nascimento dos animais nos motivou a estudar o efeito da exposição passiva à fumaça do cigarro nos pulmões e cérebro de ratos expostos desde o primeiro dia do nascimento, ou seja, durante todo o período final de desenvolvimento das estruturas pulmonares e cerebrais.

2.2. Estresse oxidativo

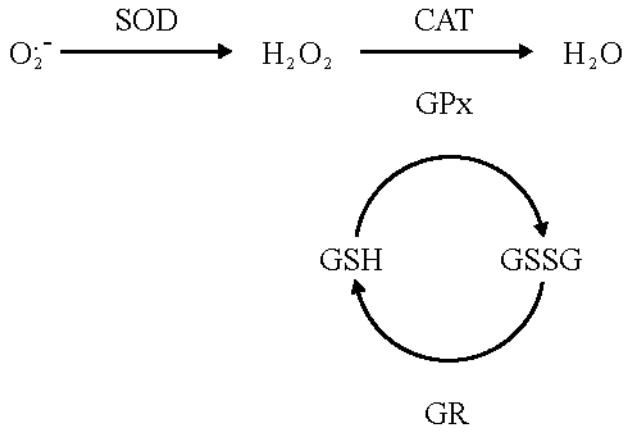
Durante o metabolismo basal das células aeróbicas existe uma produção constante de EROs, acompanhada pela sua contínua inativação através da ação de antioxidantes, de forma a manter a integridade estrutural e funcional das biomoléculas. Essas espécies reativas produzidas constantemente sob condições normais nas células eucariotas desempenham funções importantes para o organismo (Droge, 2002), tais como a proliferação, a diferenciação e a sinalização celular (Dennery, 2007).

A extensão e o tipo de dano causado pelos EROs dependem da quantidade e da natureza dos mesmos, bem como das defesas antioxidantes celulares (Davies, 1991). O desequilíbrio entre os fenômenos pró-oxidativos e as defesas antioxidantes celulares pode

desencadear mudanças fisiológicas, denominadas genericamente de estresse oxidativo (Halliwel, 1992; Dawson e Dawson, 1996). As EROs são capazes de causar um estresse oxidativo em conseqüência de suas propriedades oxidantes e da reação com os constituintes celulares (Josephy, 1997; Timbrell, 2000). A produção excessiva de EROs provoca muitas conseqüências negativas, entre elas, causa dano nas biomoléculas alvo, tais como o DNA, lipídios e proteínas (Josephy, 1997; Timbrell, 2000). Com isso, o estresse oxidativo pode estar relacionado com vários processos deletérios, tais como: mutagênese, carcinogênese, peroxidação lipídica, oxidação e fragmentação de proteínas e carboidratos (Sies, 1997). Além disso, o estresse oxidativo está relacionado com o aparecimento de diversas doenças, tais como desordens pulmonares e cardiovasculares (Klaunig e Kamendulis, 2004).

Quando as EROs reagem com os ácidos graxos insaturados nas membranas biológicas, modificam os lipídeos e a membrana perde suas características arquitetônicas, tornando-se menos firme (Josephy, 1997; Timbrell, 2000). Com isso, criam-se verdadeiras fendas iônicas que alteram sua semipermeabilidade, o que favorece a entrada e a saída indiscriminada de metabólitos e detritos da célula, provocando sua ruptura e lise com necrose (Josephy, 1997; Timbrell, 2000).

As principais EROs vinculadas ao estresse oxidativo são: o radical ânion superóxido, o radical hidroxil, o peróxido de hidrogênio, o óxido nítrico e o peroxinitrito. Estes por sua vez são neutralizados por um elaborado sistema de defesa antioxidante constituído de enzimas tais como a CAT, a SOD, a GPx e a GR (Esquema 1), além de inúmeros sistemas de defesas não-enzimáticos incluindo as vitaminas A, E e C, flavonóides, ubiquinonas e a GSH (Alexi et al., 1998; Gianni et al., 2004).



Esquema 1: Funções das enzimas antioxidantes. Radical anion superóxido ($O_2^{\bullet-}$), Peróxido de hidrogênio (H_2O_2), Água (H_2O), Glutationa oxidada (GSSG).

2.2.1. Exposição à fumaça do cigarro e estresse oxidativo

O estresse oxidativo está bastante relacionado com os danos causados pela exposição passiva à fumaça do cigarro. Sob condições fisiológicas, as células produzem EROs por meio da redução do oxigênio molecular. A produção dos derivados tóxicos de oxigênio é aumentada como resultado de vários tipos de estresse (Foyer et al., 1994). A geração de EROs, tais como o radical hidroxil ($OH^{\bullet}$), o ânion superóxido ($O_2^{\bullet-}$), o peróxido de hidrogênio (H_2O_2) e o oxigênio singleto ($^1O_2$) são um dos agentes causadores de injúria nos tecidos. Os danos causados pela exposição passiva à fumaça do cigarro estão associados com o estresse oxidativo, uma vez que o estresse oxidativo ocorre devido aos efeitos diretos das espécies reativas presentes na fumaça do cigarro (Alberg, 2002). As espécies reativas presentes na fumaça do cigarro são capazes de iniciar ou promover um dano oxidativo (Cross et al., 1993; Panda et al., 1999). De fato, o estresse oxidativo é bastante observado nos animais expostos à fumaça do cigarro (Pryor et al., 1983; Church and Pryor, 1985; Cross et al., 1993; Panda et al., 1999; Anbarasi et al., 2006; Luchese et al., 2009).

O pulmão é o primeiro órgão que entra em contato com a fumaça do cigarro, sendo altamente suscetível a geração de espécies reativas e radicais livres. O pulmão tem um grande número de defesas antioxidantes para se proteger dos danos causados pelas espécies reativas e pelos radicais livres (Morcillo et al., 1999; Foronjy e D'Armiento, 2006). Um complexo sistema de defesa antioxidante do trato respiratório consiste de defesas antioxidantes enzimáticas (GPx, GR, SOD, CAT, GST) e não-enzimáticas (GSH e ácido ascórbico), as quais são as linhas de defesa contra os oxidantes (Van der Vliet e Cross, 2000).

O cérebro também é um órgão afetado pela exposição passiva à fumaça do cigarro. O cérebro é extremamente vulnerável ao estresse oxidativo, em parte porque ele é altamente rico em ferro não ligado a hemoglobina, que é cataliticamente envolvido na produção de EROs. Além disso, o cérebro contém um grau relativamente elevado de ácidos graxos poliinsaturados que são particularmente bons substratos para as reações de peroxidação (Halliwell e Gutteridge, 2007). Neste tecido existe um pequeno número de defesas antioxidante celulares, as quais apresentam o papel de converter as espécies reativas em compostos não-reativos. Estes mecanismos de defesa incluem a SOD, GPx, CAT e mais recentemente, a família das peroxiredoxinas (Power et al., 2008).

Além disso, a exposição à fumaça do cigarro altera a atividade de enzimas antioxidantes (enzimas *scavengers* de radicais livres), que podem proteger contra os danos

celulares. Tem sido demonstrado que a exposição à fumaça do cigarro reduz a atividade de enzimas antioxidantes, tais como a CAT, SOD, GPx e GR (Anbarasi et al., 2006; Ozkan et al., 2007). Um aumento na atividade dessas enzimas antioxidantes também tem sido demonstrado após a exposição à fumaça do cigarro (Ozkan et al., 2007; Yilmaz et al., 2008; Luchese et al., 2009). Outra enzima que pode estar alterada pela exposição à fumaça do cigarro é a GST. Esta é uma enzima de metabolização de fase II (Hayes e Pulford, 2005), que cataliza a conjugação de xenobióticos com a GSH, formando um conjugado menos tóxico e mais facilmente excretado. Dessa forma, a exposição à xenobióticos, como os componentes da fumaça do cigarro, pode causar um aumento (Bindu e Annamalai, 2004; Ozkan et al., 2007; Luchese et al., 2009) ou uma redução (Ozkan et al., 2007) na atividade dessa enzima. Além disso, dados da literatura apontam a GST com uma enzima com ação antioxidante (Fiander and Schneider, 1999; Casalino et al., 2004).

A exposição à fumaça do cigarro pode, também, alterar as defesas antioxidantes não-enzimáticas como, por exemplo, a GSH, o principal tiol não-protéico nos tecidos. Trabalhos na literatura têm demonstrado que animais expostos à fumaça do cigarro apresentam uma redução no conteúdo de tióis não-protéicos (SHNP) (Anand et al., 1996; Baskaran et al., 1999; Reddy et al., 2002; Ardite et al., 2006; Anbarasi et al., 2006; Luchese et al., 2009). Outra defesa antioxidante não-enzimática alterada pela exposição à fumaça do cigarro é o ácido ascórbico que tem sido demonstrado como um agente redutor contra os oxidantes presentes na fumaça do cigarro. Tem sido demonstrada uma redução nos níveis de ácido ascórbico após a exposição à fumaça do cigarro (Ambarasi et al., 2006; Luchese et al., 2009).

2.3. Antioxidante

Tendo em vista os aspectos acima mencionados, a fumaça do cigarro pode causar um dano oxidativo nos tecidos de ratos expostos. Dessa forma, a utilização de moléculas com efeito antioxidante seria uma alternativa no tratamento dos danos causados pela exposição à fumaça do cigarro. Dados da literatura têm demonstrado a eficácia de alguns antioxidantes clássicos, tais como a vitaminas C e E, nos danos causados pela exposição passiva à fumaça do cigarro (Ozan et al., 2007; Valenca et al., 2008).

2.3.1. Selênio

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

O selênio compartilha propriedades químicas e físicas com o enxofre. Esta similaridade permite que o selênio substitua o enxofre, promovendo interações selênio-enxofre nos sistemas biológicos. Por outro lado, as diferenças nas propriedades fisico-químicas entre selênio e enxofre constituem a base de seus papéis biológicos específicos (Stadtman, 1980). Os selenóis (R-SeH) são as formas correspondentes aos tióis (R-SH), onde ocorre a substituição do átomo de S pelo átomo de Se (Klayman e Günther,1973).

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

Este calcogênio apresenta um grande número de funções biológicas, sendo a mais importante a de antioxidante. O selênio tem atividade redox e tem importância fundamental porque faz parte do sítio ativo de diversas enzimas. Já é conhecido que o selênio está presente como resíduo de selenocisteína no sítio ativo das enzimas GPx (Wingler e Brigelius-Flohé, 1999), tioredoxina redutase (Holmgren, 1985), 5'-deiodinase (Behne e Kyriakopoulos, 1990) e selenoproteína P (Ursini et al., 1990). Além disso, sabe-se que as moléculas contendo selênio, como por exemplo, o $(PhSe)_2$, podem ser melhores nucleófilos (e portanto antioxidantes) do que os antioxidantes clássicos (Arteel e Sies, 2001).

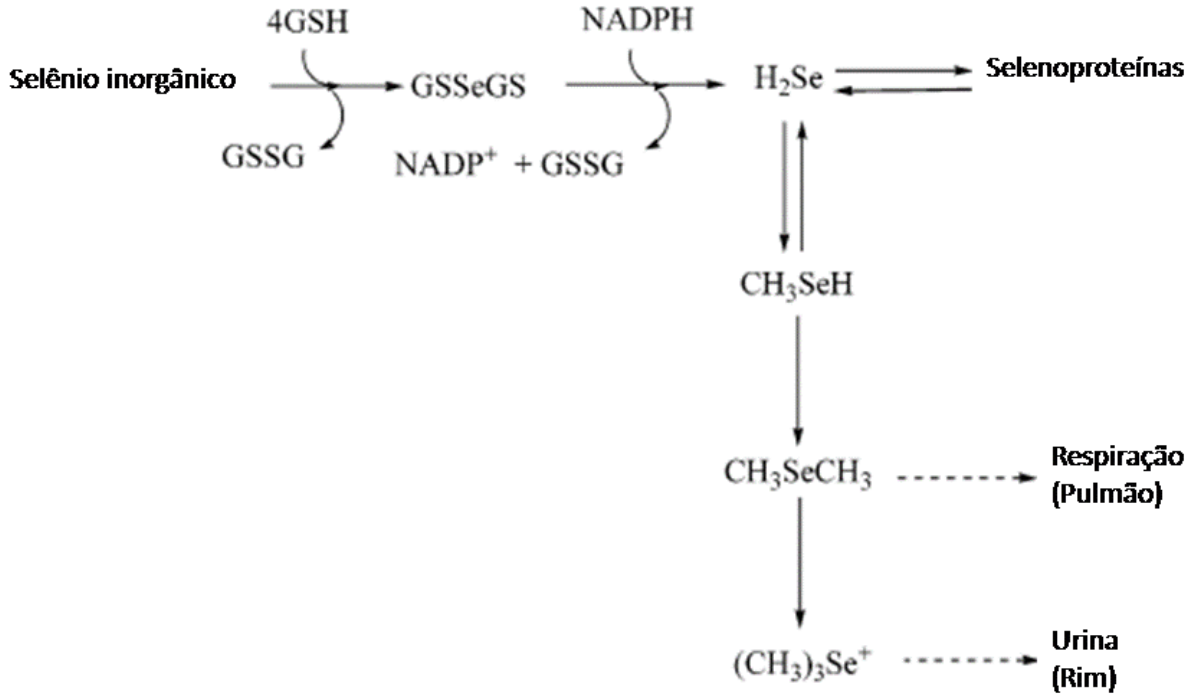
Trabalhos têm apontado que o selênio tem um papel protetor contra os efeitos danosos causados pela fumaça do cigarro (Howard et al., 1998; Van Binh et al., 2004a,b; Kosanovic e Jokanovic, 2007).

2.3.1.1. Absorção, distribuição e excreção

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

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

O selênio pode ser excretado por três vias: urina, fezes e ar expirado. A excreção urinária deste composto pode auxiliar em casos de intoxicações ou de exposições a altos níveis deste elemento (Valentine et al., 1978). Recentemente, foi demonstrado que nos níveis normais de selênio, ou seja, não tóxicos, a principal forma encontrada na urina é como seleno-açúcar. Entretanto, nos casos de doses tóxicas de selênio, o marcador biológico encontrado na urina é o trimetilselenônio (Suzuki et al., 2006). Em indivíduos expostos acidentalmente a altos níveis de selênio, pode ser realizada a detecção do composto volátil seleneto de dimetila (Mozier et al., 1988) (Esquema 2).



Esquema 2: Reações do metabolismo do selênio inorgânico. Fonte: Nogueira et al., 2004

2.3.1.2. Disseleneto de difenila $(PhSe)_2$

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), sendo importantes intermediários e reagentes muito utilizados em síntese orgânica (Paulmier, 1986; Braga et al., 1996; 1997). Conseqüentemente, o risco de contaminação ocupacional por organocalcogênios é grande. Outro aspecto relevante é a tentativa crescente de desenvolvimento de compostos organocalcogênios que possuam atividades biológicas e aplicações farmacológicas (Parnham e Graf, 1991; Nogueira et al., 2003).

Durante as últimas décadas, o interesse nos compostos orgânicos de selênio tem sido intensificado, principalmente devido ao fato de que uma variedade destes compostos possui propriedades farmacológicas (Nogueira et al., 2004). Em especial, destaca-se o $(PhSe)_2$ (Figura 1), um composto orgânico de selênio lipofílico e que apresenta inúmeras propriedades farmacológicas (Nogueira et al., 2004). De fato, estudos em animais de laboratório têm demonstrado que este composto apresenta propriedades antiúlcera (Savegnago et al., 2006), antiinflamatória e antinociceptiva (Savegnago et al., 2007), antidepressiva-*like* (Savegnago et

al., 2008, Acker et al., 2009a), ansiolítica-*like* (Savegnago et al., 2008), neuroprotetora (Ghisleni et al., 2003), anti-hiperglicêmica (Barbosa et al., 2006), pode retardar o desenvolvimento de câncer (Barbosa et al., 2008) e pode apresentar atividade complexante em animais expostos ao cádmio (Santos et al., 2005b). Além disso, o $(PhSe)_2$ apresenta efeito antioxidante em modelos de estresse oxidativo, tais como provocado por cádmio (Santos et al., 2004, 2005a,b; Borges et al., 2008), pela exposição à fumaça do cigarro (Luchese et al., 2009) e por exercício (Prigol et al., 2009a). Entretanto o exato mecanismo pelo qual esse composto apresenta efeito antioxidante ainda não foi completamente elucidado. Trabalhos na literatura sugerem que pelo fato do $(PhSe)_2$ possuir atividade semelhante a da glutationa peroxidase, este composto é um bom candidato a ser um agente antioxidante (Meotti et al., 2004; Nogueira et al., 2004). Dados da literatura indicam que o efeito do $(PhSe)_2$ em aumentar os níveis de SHNP e ácido ascórbico está envolvido na ação antioxidante do composto (Barbosa et al., 2006, 2008; Borges et al., 2008; Luchese et al., 2009).

Recentemente, nosso grupo de pesquisa tem estudado a farmacocinética do $(PhSe)_2$. Prigol et al. (2009b) demonstraram que o pico plasmático de $(PhSe)_2$ após a administração oral (500 mg/kg) foi em 30 min e a concentração plasmática máxima do composto foi de 13,13 e 10,11 μg/ml para ratos e camundongos, respectivamente. Além disso, nosso grupo de pesquisa tem trabalhado para entender se os efeitos farmacológicos do $(PhSe)_2$ estão relacionados à presença do composto ou de um metabólito ativo.

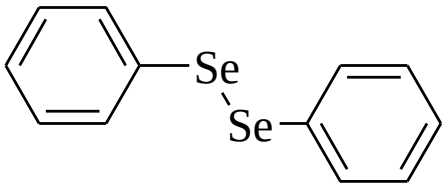


Figura 1 – Estrutura do $(PhSe)_2$.

Embora existam alguns estudos na literatura avaliando o efeito da interação entre oselênio e a fumaça do cigarro (Howard et al., 1998; Ozkan et al., 2007), estudos utilizando o $(PhSe)_2$ não existem. Em um estudo com ratos adultos, verificamos que o $(PhSe)_2$ restaurou o dano oxidativo provocado pela exposição passiva à fumaça do cigarro nos pulmões e cérebros de ratos (Luchese et al., 2009), sugerindo o efeito benéfico deste organocalcogênio no estresse oxidativo causado pela fumaça do cigarro .

2.4. Mecanismos antioxidantes

2.4.1. Dehidroascorbato (DHA) redutase

2.4.1.1. Ácido ascórbico

O ácido ascórbico, uma vitamina solúvel em água, é um nutriente essencial para o homem, macacos e porcos da índia, os quais não são capazes de sintetizar este composto (Burns, 1957). O ácido ascórbico tem diversas funções no corpo, tais como cofator na biosíntese enzimática de colágeno, carnitina, catecolamina e neurohormônios peptídeos (Burns, 1957; Levine, 1986; Wilson, 2002). O ácido ascórbico também é um antioxidante que reduz as EROs e as ERNs a moléculas estáveis (Bendich et al., 1986; Meister, 1994; Winkler et al., 1994). Em todas essas funções do ácido ascórbico, o DHA é formado diretamente ou via desprotonação dos radicais ascorbil (Bendich et al., 1986).

2.4.1.2. Transporte de ácido ascórbico e de DHA

O ácido ascórbico é transportado através das membranas celulares por dois mecanismos distintos. Um sistema de transporte tem absoluta especificidade por ácido ascórbico, e um segundo sistema que tem absoluta especificidade por DHA (Rivas et al., 2008). Ou seja, quando o ácido ascórbico não for transportado por seu transportador específico, este ácido ascórbico presente do lado de fora das células pode ser oxidado a DHA, o qual será transportado por um mecanismo diferente (Vera et al., 1994; Welch et al., 1995).

O ácido ascórbico é transportado por uma família de transportadores saturáveis dependentes de sódio (SVCT) (Thorn et al., 1991; Dixon e Wilson, 1992; Welch et al., 1993,1995; Bergsten et al., 1995; Rivas et al., 2008). O transporte de DHA ocorre através dos transportadores de glicose (GLUT), principalmente três isoformas: GLUT 1, GLUT 3 e GLUT 4 (Rumsey et al., 1997; Rivas et al., 2008). Os GLUT são capazes de transportar o

DHA porque ele apresenta uma estrutura muito semelhante à estrutura da glicose (Rumsey et al., 1997) (Figura 2).

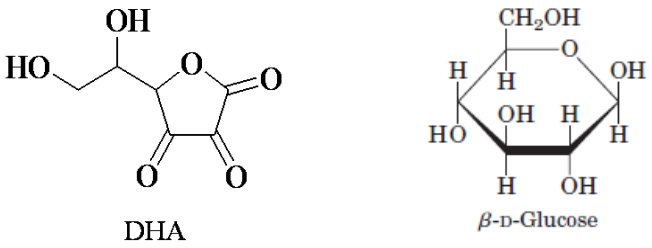
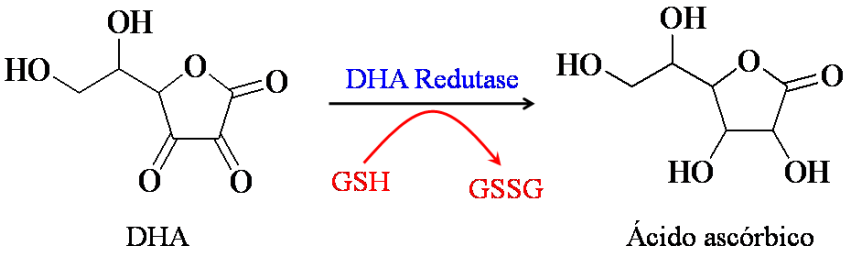


Figura 2 - Estrutura do DHA e da glicose.

2.4.1.3. Função da DHA Redutase

As células dos mamíferos eficientemente transportam e reduzem DHA a ácido ascórbico e, portanto, essa reciclagem leva ao acúmulo de ácido ascórbico nos tecidos (Welch et al., 1995; Wilson, 2002). A DHA redutase é a enzima que catalisa a redução do DHA a ácido ascórbico, com o consumo de GSH (Wells et al., 1995; Xu et al., 1996) (Esquema 3).



Esquema 3 – Conversão do DHA a ácido ascórbico pela ação da DHA redutase.

Do ponto de vista celular, é muito mais vantajoso reduzir o DHA a ácido ascórbico do que sintetizar o ácido ascórbico novamente. Esse mecanismo de reciclagem do ácido ascórbico é importante em espécies que não são capazes de sintetizar este antioxidante, como é o caso dos seres humanos (Banhegyi et al., 1997).

O efeito mimético da atividade da DHA redutase pode estar relacionado com o mecanismo antioxidante do ebselen, um composto orgânico de selênio com propriedades farmacológicas já consolidadas na literatura. De acordo, Jung et al. (2002) demonstraram que o ebselen apresenta atividade mimética da DHA redutase, e esse mecanismo pode servir para explicar as propriedades antioxidantes e outros efeitos farmacológicos do composto. Já que, a redução do DHA a ácido ascórbico no interior das células, aumenta a concentração do ácido ascórbico nos tecidos e assim, aumenta o poder antioxidante contra o estresse oxidativo (Banhegyi et al., 1997).

2.4.2. GST

A GST é uma enzima de fase II que está amplamente distribuída em diversos tecidos. Essa enzima promove a conjugação de GSH com uma grande variedade de compostos eletrofílicos, resultando na formação de substâncias que são facilmente excretadas do corpo (Chasseaund, 1979; Cervello et al., 1992). Nos ensaios para a determinação da atividade da GST, utiliza-se a GSH e um substrato sintético (CDNB) (Esquema 4). Além disso, autores têm demonstrado que a GST é uma defesa antioxidante enzimática e serve para proteger os tecidos contra o estresse oxidativo (Mosialou e Morgenstern, 1989; Hayes e Pulford, 1995; Fiander e Schneider, 1999).

Esquema 4 – Conjugação do CDNB com GSH catalisada pela GST. 1-Cloro-2,4-dinitrobenzeno (CDNB)

Como a GST apresenta ação antioxidante, um estudo utilizando o ebselen, um composto orgânico de selênio, demonstrou que o efeito mimético da atividade dessa enzima pode estar relacionado com o mecanismo antioxidante deste composto, e dessa forma, explicar as propriedades antioxidantes e outros efeitos farmacológicos do ebselen (Jung et al., 2002).

2.4.3. *Scavenger*

Como já se sabe, os radicais livres causam peroxidação lipídica nos tecidos. Dessa forma, substâncias *scavenger* de radicais livres podem proteger contra as reações de peroxidação, e assim, proteger do estresse oxidativo (Soares et al., 1997). Os ensaios utilizando radicais 1,1-difenil-2-picril-hidrazil (DPPH) e 2,2'-azino-bis(3-etilbenztiazolina-6-ácido sulfônico) (ABTS) estão entre os métodos mais utilizados e mais populares para a determinação da capacidade e dos mecanismos antioxidantes. Diversos trabalhos da literatura têm demonstrado o efeito *scavenger* de radicais DPPH e ABTS de substâncias naturais derivadas de frutas, vegetais e ervas (Ak e Gulcin, 2008; Koksal e Gulcin, 2008; Koksal et al., 2009), da adrenalina (Gulcin, 2009), e de compostos sintéticos, tais como compostos orgânicos de selênio (Acker et al., 2009b) e telúrio (Acker et al., 2009b; Souza et al., 2009).

Quando um antioxidante é adicionado a um meio com os radicais, há um grau de descoloração devido à presença dos antioxidantes que revertem a formação do radical $DPPH^{\bullet}$ e do radical cátion $ABTS^{\bullet+}$ (Esquema 5).

$$DPPH^{\bullet} + AH \rightarrow DPPH_2 + A^{\bullet}$$

$$ABTS^{\bullet+} + AH \rightarrow ABTS^{+} + A^{\bullet}$$

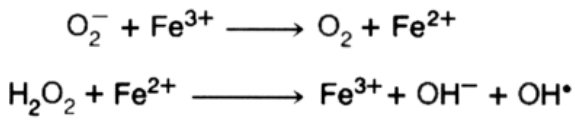
Esquema 5 – Estabilização dos radicais pelos antioxidantes.

2.3.4. Auto-oxidação do Fe^{2+}

A formação de EROs está intimamente relacionada com o estado redox de metais de transição, tais como ferro (Halliwell e Gutteridge, 1990). O íon ferroso (Fe^{2+}) pode facilitar a produção de EROs nos sistemas animais e humanos, e a capacidade das substâncias de proteger a auto-oxidação do Fe^{2+} pode ser um mecanismo antioxidante delas (Puntel et al., 2008, 2009; Khomenko et al., 2009).

O ânion superóxido é rapidamente produzido através da redução de um elétron do oxigênio pelo íon ferroso, e é dismutado a peróxido de hidrogênio por mecanismos

enzimáticos e não-enzimáticos (Fridovich, 1989). O peróxido de hidrogênio é então convertido no radical hidroxil pela reação de Fenton, o qual requer ferro reduzido (Fe^{2+}) (Halliwell e Gutteridge, 1990) (Esquema 6). Além disso, o Fe^{2+} pode ligar-se ao oxigênio molecular, e formar o íon perferril (Fe^{2+}-O_2) (Miller e Aust, 1989). O radical hidroxil e o íon perferril são altamente reativos. Eles iniciam a peroxidação lipídica celular, e com isso, contribuem para o estresse oxidativo (Fridovich, 1989; Hippeli e Elstner, 1999). A inibição eficaz da auto-oxidação do Fe^{2+} mediada por isocitrato resulta na diminuição da formação dos íons perferril, mantendo o ferro no estado reduzido. Dados na literatura demonstram que a proteção da auto-oxidação do Fe^{2+} está relacionada ao mecanismo antioxidante de substâncias, tais como compostos orgânicos de selênio e telúrio (Acker et al., 2009b) e compostos fenólicos derivados de plantas (Ak e Gulcin, 2008).



Esquema 6 - Seqüência da reação de Fenton.

Considerando que o $(PhSe)_2$ apresenta propriedade antioxidante e que os mecanismos envolvidos na ação do composto não foram completamente elucidados, isso nos motivou a estudar os possíveis mecanismos envolvidos na ação antioxidante deste composto.

3. OBJETIVOS

3.1. Objetivo geral

A fumaça do cigarro é uma mistura complexa de diversos constituintes identificados, entre eles, substâncias reativas, as quais são capazes de iniciar ou promover um dano oxidativo. O fato do $(PhSe)_2$ apresentar efeito antioxidante associado ao uso de antioxidantes para proteger do dano oxidativo motivou este trabalho. O objetivo geral deste trabalho foi verificar o efeito antioxidante e os mecanismos envolvido na ação do $(PhSe)_2$.

3.2. Objetivos específicos

- Verificar o efeito antioxidante do $(PhSe)_2$ no dano oxidativo induzido pela exposição passiva à fumaça do cigarro nos pulmões e cérebro de ratos jovens em dois protocolos experimentais;

- Investigar o efeito mimético do $(PhSe)_2$ na atividade das enzimas DHA redutase e GST;

- Analisar a atividade do $(PhSe)_2$ como *scavenger* de radicais $DPPH^{\bullet}$ e $ABTS^{\bullet+}$.

- Avaliar o papel protetor do $(PhSe)_2$ na auto-oxidação do Fe^{2+}.

- Estudar o envolvimento da síntese da glutationa no efeito antioxidante do $(PhSe)_2$, utilizando para isto a butionina sulfoximina (BSO).

4. ARTIGOS E MANUSCRITO CIENTÍFICOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos científicos, manuscritos e anexo, os quais se encontram assim 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 nas revistas científicas (artigos 1, 2 e 3). O manuscrito 1 está disposto da mesma forma que foi submetido para avaliação.

4.1. Artigo 1:

Disseleneto de Difenila Previne o Dano Oxidativo Induzida pela Exposição à Fumaça do Cigarro no Pulmão de Ratos Jovens

DIPHENYL DISELENIDE PREVENTS OXIDATIVE DAMAGE INDUCED BY CIGARETTE SMOKE EXPOSURE IN LUNG OF RAT PUPS

Cristiane Luchese, Eluza C. Stangherlin, Ana P. Ardais, Cristina W. Nogueira, Francielli W. Santos

Toxicology 230 (2007) 189-196

Available online at www.sciencedirect.com
ScienceDirect
Toxicology 230 (2007) 189–196
TOXICOLOGY
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Diphenyl diselenide prevents oxidative damage induced by cigarette smoke exposure in lung of rat pups

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Received 18 August 2006; received in revised form 8 November 2006; accepted 12 November 2006
Available online 19 November 2006

Abstract

The effect of cigarette smoke exposure on lungs of rat pups was evaluated. Animals were exposed to passive cigarette smoke during 3 weeks and a number of toxicological parameters in lung of pups were examined, such as lipid peroxidation, δ-aminolevulic acid dehydratase (δ-ALA-D) activity, components of the enzymatic antioxidant defenses (superoxide dismutase (SOD) and catalase activities) and non-enzymatic antioxidant defenses (Vitamin C and non-protein thiol (NPSH) levels). Furthermore, a possible protective effect of diphenyl diselenide, $(PhSe)_2$, was studied. The results demonstrated an increase in lipid peroxidation, an inhibition of δ-ALA-D activity, a reduction of Vitamin C and NPSH levels induced by cigarette smoke exposure, indicating damage in lungs of rat pups. Oral administration of $(PhSe)_2$ (0.5 mg/kg) restored TBARS levels, non-enzymatic antioxidant defenses and activity of δ-ALA-D. These results indicated that exposure to cigarette smoke enhanced oxidative stress, thereby disturbing the tissue defense system. $(PhSe)_2$ protected against oxidative damage induced by cigarette smoke exposure in lung of rat pups.

Keywords: Diphenyl diselenide; Selenium; Cigarette smoking; Lungs; Oxidative stress; Antioxidant

1. Introduction

Cigarette smoking has been implicated as a major risk factor in the development of pulmonary, cardio and cerebrovascular diseases, cancers and several others (US DHHS, 1989). Cigarette smoking is a complex mixture of over 4000 identified constituents (Rahman et al., 1996; Genbacev-Krtolica, 2005) that include numerous reactive substances such as a large quantity of reactive aldehydes (Park et al., 1998), free radical species, such as oxygen free radicals and nitrogen species (Pryor and Stone, 1993), and diverse metals such as cadmium (Cd^{2+}) (WHO, 1992). Free radicals and other reactive oxygen and nitrogen species (ROS

Abbreviations: δ-ALA-D, δ-aminolevulinic acid dehydratase; δ-ALA, δ-aminolevulinic acid; SOD, superoxide dismutase; NPSH, non-protein thiols; $(PhSe)_2$, diphenyl diselenide; TBARS, thiobarbituric acid reactive species; ROS, reactive oxygen species; NOS, reactive nitrogen species; GSH, reduced glutathione; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); H_2O_2, hydrogen peroxide; SDS, sodium dodecil sulphate; TBA, tiobarbituric acid

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doi:10.1016/j.tox.2006.11.051

and NOS, respectively) are capable of initiating or promoting oxidative damage (Schumacher et al., 1977; Pryor et al., 1983; Church and Pryor, 1985; Cross et al., 1993; Panda et al., 1999).

The oxidative effects of ROS are controlled by exogenous antioxidants such as Vitamins E and C, and also by endogenous antioxidants such as scavenger enzymes (superoxide dismutase (SOD) and glutathione peroxidase) (Halliwell and Gutteridge, 2000). In fact, some studies have suggested a protective role for vitamins and antioxidants in modifying the major diseases related to cigarette smoking (Tiwari, 2004; Anbarasi et al., 2005). Therefore, the concept that selenium-containing molecules may be better nucleophiles (and therefore antioxidants) than classical antioxidants, has led to design of synthetic organoselenium compounds (Arteel and Sies, 2001). In addition, diphenyl diselenide, $(PhSe)_2$, retards the lipid peroxidation induced by a variety of oxidants (Rossato et al., 2002). Moreover, this organochalcogen has been shown to be better antioxidant (Meotti et al., 2004) and less toxic to rodents than other selenium compounds (Nogueira et al., 2003; Meotti et al., 2003).

Passive maternal exposure to tobacco smoke (involuntary maternal smoking) during pregnancy is also associated with an increased incidence of asthma and a risk for patterns of negative developmental outcomes (Barber et al., 1996). Passive smoking and tobacco exposure through breast milk increase the risk of sudden infant death syndrome (Klonoff-Cohen et al., 1995). In addition, children are the most susceptible group for environmental tobacco smoke exposure because their bronchial tubes are smaller and their immune systems are less developed respiratory and aural consequences if exposed to cigarette smoke (WHO, 1999; Tutka et al., 2002).

Therefore, the objective of the present study was to determine the effect of passive cigarette smoke exposure on lungs of rat pups. The possible protective effect of diphenyl diselenide, an antioxidant agent, on damage induced by cigarette smoke was also evaluated.

2. Materials and methods

2.1. Chemicals

δ-Aminolevulinic acid (δ-ALA) and *p*-dimethylaminobenzaldehyde were purchased from Sigma (St. Louis, MO, USA). Diphenyl diselenide $(PhSe)_2$ was prepared according to Paulmier (1986). All other chemicals were of analytical grade and obtained from standard commercial suppliers. $(PhSe)_2$ was dissolved in canola oil which was obtained from a standard commercial supplier. Cigarettes of diverse commercial marks containing 0.8 mg of nicotine were used.

2.2. Animals

Virgin female Wistar rats (180–240 g) from our own breeding colony were used. The animals were kept on a 12-h light:12-h dark cycle, at a room temperature of 22 ± 2 °C, with free access to food and water. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, School of Veterinary Medicine and Animal Science of the University of Sao Paulo, Brazil. Sexually naive female rats were mated with male previously tested as fertile (three females and one male in each cage). The onset of pregnancy was confirmed by the presence of sperm in vaginal smears (day 0 of pregnancy) and pregnant dams were immediately housed in individual cages.

At birth, all litters were culled to eight pups. Whenever possible, only male rats were kept within the litter. Female rats were kept just to maintain equal litter sizes. Each litter presented pups (female and male) representative from each group of treatment.

2.3. Cigarette smoke exposure

Groups of eight male or female suckling rats, initially weighting 5–6 g, were exposed to the smoke of commercial cigarettes during 3 weeks (lactational period). Pups were exposed to cigarette smoke in whole body smoke exposure chamber. In the first week, animals were exposed to the smoke of one cigarette. In the second week, animals were concomitantly exposed to the smoke of two cigarettes. In the third week, animals were concomitantly exposed to the smoke of three cigarettes. The duration of each exposure was 15 min. Before each smoke exposure, animals received an oral administration of diphenyl diselenide (0.5 mg/kg, 2 μl of volume) or canola oil (2 μl of volume). Control animals followed the same procedure except the cigarette smoke exposure (Table 1).

At the end of experimental period (3 weeks), animals were killed by decapitation and lung tissues were immediately excised. The lungs were washed on ice-cold saline and rapidly homogenized in 50 mM Tris–HCl, pH 7.5 (1/5, w/v) and centrifuged at 2400 × *g* for 15 min at 4 °C.

Table 1
Protocol of rat pups exposure

Groups	Treatments
Group 1	Canola oil (oral) + without cigarette smoke exposure
Group 2	Diphenyl diselenide (oral) + without cigarette smoke exposure
Group 3	Canola oil (oral) + cigarette smoke exposure
Group 4	Diphenyl diselenide (oral) + cigarette smoke exposure

Animals were exposed to cigarette smoke during 3 weeks (lactation period), 7 times/week.

2.4. Determination of the body weight

The body weight gain of pups was monitored for the whole course of the experiment.

2.5. Lipid peroxidation

Thiobarbituric acid reactive species (TBARS) were determined as described by Ohkawa et al. (1979). An aliquot (300 μl) of lung tissue was incubated with thiobarbituric acid (TBA) 0.8% (500 μl), acetic acid buffer (500 μl), pH 3.4, and sodium dodecil sulphate (SDS) 8.1% (200 μl) at 95 °C for 2 h. The color reaction was measured at 532 nm.

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

Pulmonary δ-ALA-D activity was assayed according to the method of Sassa (1982) by measuring the rate of product (porphobilinogen) formation except that 1 M potassium phosphate buffer, pH 6.8 (75 μl), and 12 mM ALA (100 μl) were used. An aliquot of 300 μl of homogenized tissue was incubated for 2 h at 37 °C. The reaction was linear in relation to protein and time of incubation. The reaction product was determined using modified Erlich's reagent (1 ml) at 555 nm.

2.7. Superoxide dismutase activity

Superoxide dismutase activity in lung homogenate was assayed spectrophotometrically as described by Misra and Fridovich (1972). This method is based on the capacity of SOD in inhibiting autoxidation of adrenaline to adrenochrome. The color reaction was measured at 480 nm. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50% at 26 °C. The lung tissue was diluted 1:10 (v/v) for determination of SOD activity in test day. Aliquots (10, 20 and 50 μl) of lung tissues were added in a glycine buffer 50 mM (1 ml), pH 10.3. Enzymatic reaction was started by adding epinephrine (17 μl).

2.8. Catalase activity

The catalase activity was assayed spectrophotometrically by the method of Aebi (1984), which involves monitoring the disappearance of H_2O_2 in the presence of lung homogenate at 240 nm. An aliquot (20 μl) of lung tissue was added in potassium phosphate buffer 50 mM (1910 μl), pH 7.0, and the enzymatic reaction was initiated by adding H_2O_2 (70 μl). The enzymatic activity was expressed in Units (1 U decomposes 1 μmol H_2O_2/min at pH 7 at 25 °C).

2.9. Determination of non-protein thiols (NPSH)

NPSH in lung were determined by the method of Ellman (1959). To determine NPSH in lungs of pups, the homogenate was centrifuged at 4000 × *g* at 4 °C for 10 min and the supernatant (500 μl) was mixed (1:1) with 10% trichloroacetic acid (500 μl). After the centrifugation, the protein pellet was discarded and free -SH groups were determined in the clear supernatant. An aliquot (200 μl) of supernatant was added in potassium phosphate buffer 1 M (750 μl), pH 7.4, and 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) 10 mM (50 μl). The color reaction was measured at 412 nm.

2.10. Ascorbic acid determination

Ascorbic acid determination was performed as described by Jacques-Silva et al. (2001). Protein (lung) was precipitated in 10 volumes of a cold 4% trichloroacetic acid solution. An aliquot of the sample in a final volume of 1 ml of the solution was incubated for 3 h at 38 °C then 1 ml H_2SO_4 65% (v/v) was added to the medium. The reaction product was determined using color reagent containing 4.5 mg/ml dinitrophenyl hydrazine and $CuSO_4$ (0.075 mg/ml). The color reaction was measured spectrophotometrically at 520 nm.

2.11. Protein determination

Protein was measured by the Coomassie blue method according to Bradford (1976) using bovine serum albumin as standard. An aliquot (50 μl) of lung tissue diluted 1:10 (v/v) was added in Coomassie blue reactive (2.5 ml). The color was measured spectrophotometrically at 595 nm.

2.12. Statistical analysis

Data are expressed as mean ± S.D. Statistical analysis was performed using a two-way ANOVA followed by the Duncan's test. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Body weight

There was no change in the body weight gain of pups in none of experimental groups during the period of exposure (data not shown).

3.2. Lipid peroxidation

Two-way ANOVA of TBARS levels yielded a significant cigarette smoke exposure × $(PhSe)_2$ interaction ($F_{1,17} = 8.7612$; $p < 0.01$). Post hoc comparisons demonstrated that the cigarette smoke exposure increased lipid peroxidation in lung of pups (1.3 times higher than in the control group) ($p < 0.05$). $(PhSe)_2$ treatment was effective in restoring the enhance of TBARS levels caused by cigarette smoke exposure in lung of pups (Fig. 1).

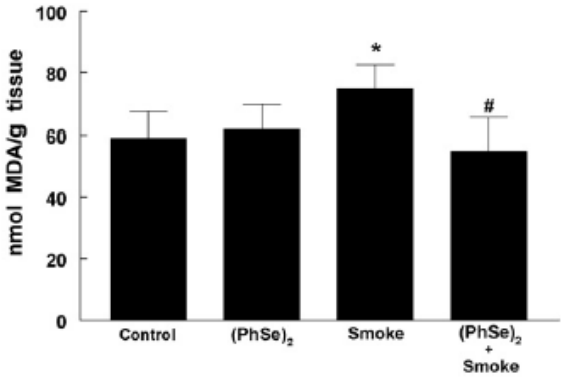

Fig. 1. Effect of $(PhSe)_2$ on TBARS levels in lung of pups after cigarette smoke exposure. Data are reported as mean ± S.D. of five animals per group and expressed as nmol MDA (malondialdehyde)/g tissue. (*) Denoted $p < 0.05$ as compared to the control group (two-way ANOVA/Duncan). (#) Denoted $p < 0.05$ as compared to the smoke group (two-way ANOVA/Duncan).

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

Two-way ANOVA of δ-ALA-D activity revealed a significant main effect of cigarette smoke exposure ($p < 0.05$). Post hoc comparisons demonstrated that cigarette smoke exposure significantly inhibited δ-ALA-D activity (14.8% of inhibition) in lung of pups ($p < 0.05$). $(PhSe)_2$ restored the inhibition caused by cigarette smoke exposure in enzyme activity (Fig. 2).

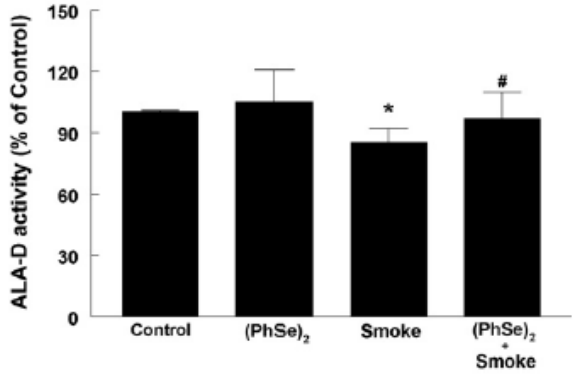

Fig. 2. Effect of $(PhSe)_2$ on δ-ALA-D activity in lung of pups after cigarette smoke exposure. Lung tissue was pre-incubated at 37 °C for 10 min. Enzymatic reaction was initiated by adding the substrate (ALA) to a final concentration of 2.2 mM in a medium containing 45 mM phosphate buffer, pH 6.8. Data are reported as mean ± S.D. of eight animals per group and expressed as % of control. δ-ALA-D activity of control (100%) was of 12.18 ± 1.66 nmol of porphobilinogen/mg protein/h. (*) Denoted $p < 0.05$ as compared to the control group (two-way ANOVA/Duncan). (#) Denoted $p < 0.05$ as compared to the smoke group (two-way ANOVA/Duncan).

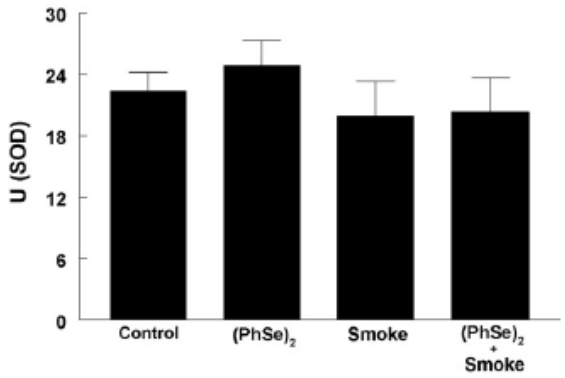

Fig. 3. Effect of $(PhSe)_2$ on superoxide dismutase in lung of pups after cigarette smoke exposure. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50% at 26 °C. Data are reported as mean ± S.D. of eight animals per group.

3.4. Superoxide dismutase activity

Two-way ANOVA of SOD activity demonstrated that exposure to cigarette smoke did not alter the enzyme activity in lung of pups (Fig. 3).

3.5. Catalase activity

Two-way ANOVA of catalase activity revealed exposure to cigarette smoke did not change the enzyme activity in lung of pups (Fig. 4).

3.6. Determination of non-protein thiols

Two-way ANOVA of NPSH levels yielded a significant cigarette smoke exposure × $(PhSe)_2$ interaction

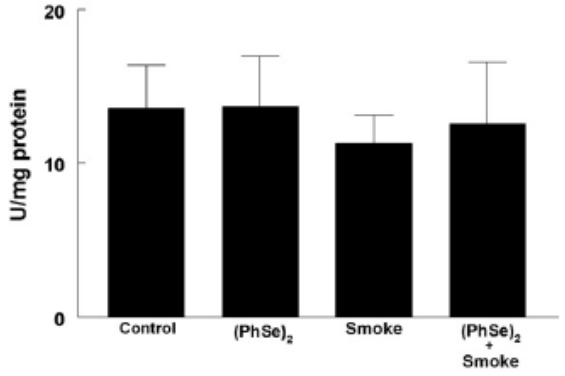

Fig. 4. Effect of $(PhSe)_2$ on catalase activity in lung of pups after cigarette smoke exposure. One unit of enzyme was defined as the amount of enzyme required for monitoring the disappearance of H_2O_2. The enzymatic activity was expressed in Units (1 U decomposes 1 μmol H_2O_2/min at pH 7 at 25 °C). Data are reported as mean ± S.D. of eight animals per group.

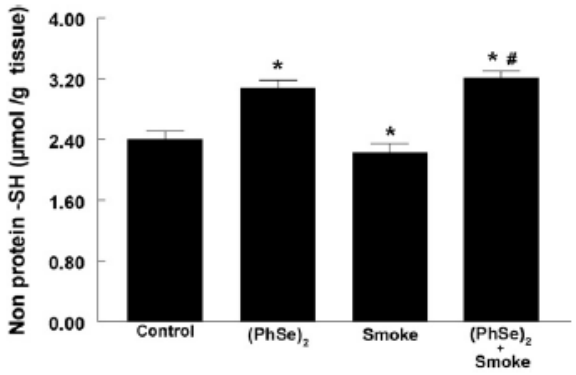

Fig. 5. Effect of $(PhSe)_2$ on non-protein thiol groups content in lung of pups after cigarette smoke exposure. Data are reported as mean ± S.D. of five animals per group. (*) Denoted $p < 0.05$ as compared to the control group (two-way ANOVA/Duncan). (#) Denoted $p < 0.05$ as compared to the smoke group (two-way ANOVA/Duncan).

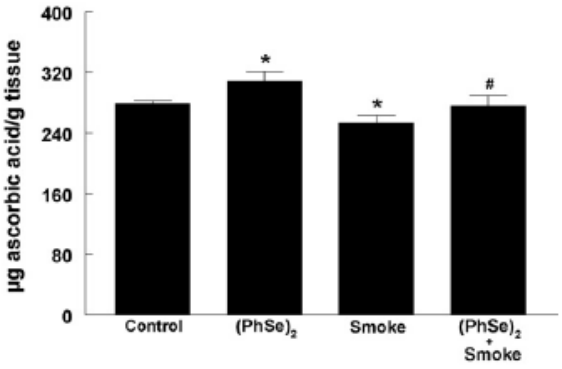

Fig. 6. Effect of $(PhSe)_2$ on ascorbic acid levels in lung of pups after cigarette smoke exposure. Data are reported as mean ± S.D. of eight animals per group. (*) Denoted $p < 0.05$ as compared to the control group (two-way ANOVA/Duncan). (#) Denoted $p < 0.05$ as compared to the smoke group (two-way ANOVA/Duncan).

($F_{1,16} = 12.26$; $p < 0.05$). Post hoc comparisons demonstrated that exposure to cigarette smoke reduced NPSH levels (7.5%) in lung of pups ($p < 0.05$). $(PhSe)_2$ increased per se NPSH levels (28.3%) in lung of pups ($p < 0.05$). $(PhSe)_2$ treatment restored NPSH levels diminished by cigarette smoke exposure in lung of pups (Fig. 5).

3.7. Ascorbic acid determination

Two-way ANOVA of ascorbic acid content yielded a significant main effect of cigarette smoke exposure ($p < 0.0001$). Post hoc comparisons demonstrated that exposure to cigarette smoke exposure significantly reduced ascorbic acid levels (9.2%) in lung of pups ($p < 0.0001$). $(PhSe)_2$ presented a significant main effect on ascorbic acid levels ($p < 0.001$). This compound significantly increased per se ascorbic acid levels (10.6%, $p < 0.001$). $(PhSe)_2$ ameliorated ascorbic acid levels reduced by cigarette smoke exposure in lung of pups (Fig. 6).

4. Discussion

Passive smoking is an inset risk or exacerbation factor for various children's diseases, such as bronchial asthma, lower respiratory illness, middle ear disease, and sudden infant death syndrome (Hofhuis et al., 2003). Children are more susceptible than adults to cigarette smoke because children have higher respiratory rates and a different metabolism compared to adults (Dejin-Karlsson et al., 1998). The current study found that passive cigarette smoke exposure induces oxidative stress in lung of rat pups not only by augmenting lipid peroxidation but also by inhibiting δ-ALA-D activity and by reducing non-enzymatic antioxidant status. The data presented here are also consistent with the antioxidant effect of diphenyl diselenide demonstrated by the reduction of peroxidative changes caused by cigarette smoke exposure.

Regardless of TBARS levels, an increase in lipid peroxidation was observed after cigarette smoke exposure and diphenyl diselenide was effective in improving TBARS status towards the normal level. Our research group has reported that diphenyl diselenide is a potent antioxidant agent (Nogueira et al., 2004) and presents higher thiol peroxidase activity than other diselenides in different tissues (Wilson et al., 1989; Meotti et al., 2004). In this study, we demonstrated for the first time an antioxidant effect of diphenyl diselenide in lung of rat pups. Accordingly, lung has been reported as a target of selenium compounds in some experimental protocols (El-Bayoumy, 2001).

The results presented above are in accordance with the inhibition of δ-ALA-D activity in lung of pups exposed to cigarette smoke found in the current study. Tentatively, the inhibition of δ-ALA-D activity by cigarette smoke exposure could be related to an increase in lung lipid peroxidation. In fact, other authors (Pande and Flora, 2002) and we (Folmer et al., 2002; Soares et al., 2003; Nogueira et al., 2004) have reported δ-ALA-D as an enzyme extremely sensitive to situations associated with oxidative stress in liver, blood, kidney and brain of rats. For this ending point of toxicity $(PhSe)_2$ was effective in restoring the enzyme activity. Taking these results in consideration, the effect caused by $(PhSe)_2$ in restoring δ-ALA-D activity and in ameliorating TBARS levels is related to the antioxidant potential of this compound, which has been reported by us (Nogueira et al., 2004).

Our data point out no change in superoxide dismutase and catalase activities after cigarette smoke exposure in rat pups. It is difficult clearly to explain why the activities of SOD and catalase were not changed by smoke exposure. Probably the schedule of protocol exposure is the responsible for the lack of alteration in the activity of antioxidant enzymes. Since our experimental protocol was carried out by 3 weeks, it is possible that the time of exposure had been insufficient to alter the enzymes activities. Conversely, other authors demonstrated that exposure to cigarette smoke enhances the production of antioxidant enzymes as a result of adaptive response, which consequently mitigate the damage caused by cigarette smoke (Hilbert and Mohsenin, 1996). On the other hand, our findings are in agreement with the lack of alteration in SOD after nicotine administration (Husain et al., 2001) and catalase after smoke exposure (Tomaki et al., 2006) in lung. In addition, Baskaran et al. (1999) reported an increase in activity of antioxidant enzymes only after 4 weeks of exposure to cigarette smoke. Therefore, the controversy of these data is probably because of the differences in experimental protocols. In fact, we verified the effect of cigarette smoke after 3 weeks of smoke exposure.

Authors have linked antioxidants, both endogenous and dietary, as playing important roles in preventing lung injury from exposure to various environmental toxicants (Cross et al., 2002; Ho, 2002). Smith et al. (2002) have reported that non-enzymatic antioxidant defenses (glutathione, Vitamin C) are involved in protecting against the injurious effects of ROS.

In this study, the levels of NPSH reduced in lung of pups after cigarette smoke exposure could be associated to the elevation in lipid peroxidation levels. Diphenyl diselenide restored NPSH levels, an antioxidant defense, in lung of pups reinforcing the role of this organoselenium compound against oxidative stress. Accordingly, various experimental studies (Anand et al., 1996; Baskaran et al., 1999; Reddy et al., 2002; Ardite et al., 2006) have demonstrated that exposure to cigarette smoke induced depletion of NPSH in lung. The depletion was directly associated with the elevation of lipid peroxidation which could be attributed to its protection against ROS generated by smoke (Baskaran et al., 1999). Rats exposed to cigarette smoke presented depletion in the levels of Vitamin C (Anbarasi et al., 2006). In agreement, our results showed a decrease in Vitamin C levels in lung of pups exposed to cigarette smoke. Of note, Vitamin C has been reported as the first strong reductant with cigarette smoke oxidants affording considerable protection to the cells (Kallner et al., 1981). Diphenyl diselenide restored Vitamin C levels supporting the ability of this organoselenium compound in reducing the oxidative stress in lungs.

Oxidative stress, provoked by tobacco, causes redox instability and activation of body protective mechanism, which results in a decrease in the concentration of some antioxidants (Sobczak et al., 2004; Avti et al., 2006). Thus, the changes observed in the non-enzymatic antioxidant defenses (NPSH and Vitamin C) after cigarette smoke exposure could be due to the adaptative response of pulmonary tissue. Moreover, this may in part reflect the participation of these antioxidants in toxicological mechanism by which cigarette smoke exposure causes damage in lung of pups. The lack of changes in the body weight gain in this experimental protocol reinforces the perspective of the use of this compound as a possible alternative in prevention of oxidative damage induced by cigarette smoke exposure in pulmonary tissue. Although the effects of smoke are discrete, it is important to point out that all other results obtained are indicative of oxidative stress. In fact, the parameters of oxidative stress evaluated, with an exception of catalase and SOD activities, were significantly changed by smoke exposure, reinforcing the consistency of the data obtained.

The above findings show that passive cigarette smoke exposure induces oxidative stress in lung of pups by augmenting lipid peroxidation and diminishing δ-ALA-D activity and non-enzymatic antioxidant status. Diphenyl diselenide restored the changes induced by cigarette smoke exposure in pulmonary tissue. Therefore, the results of the current investigation suggest that diphenyl diselenide is a potent antioxidant against toxicity induced by passive smoke in lung, an organ highly prone to oxidative stress. In addition, the dose of diphenyl diselenide used in this study (10 mg/kg) did not compare to that which demonstrated by Meotti et al. (2003). The oral dose is about 37-times lower than LD_{50} to rats (374.4 mg/kg) administered intraperitoneally and did not induce hepatic and renal toxicity (Meotti et al., 2003). Thus, based on toxicological studies of our group, we believe that the dose of diphenyl diselenide utilized in rats presents a good margin of safety. However, we cannot extrapolate our findings to human and further studies are warranted to elucidate the precise mechanism of diphenyl diselenide effect.

Acknowledgements

The financial support by FAPERGS, CAPES and CNPq is gratefully acknowledged. C.W.N. and F.W.S. are recipients of CNPq fellowships.

References

Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 105, 121–126.
Anand, C.V., Anand, U., Agarwal, R., 1996. Anti-oxidant enzymes, gammaglutamyl transpeptidase and lipid peroxidation in kidney of rats exposed to cigarette smoke. Indian J. Exp. Biol. 34, 486–488.
Anbarasi, K., Sabitha, K.E., Devi, C.S.S., 2005. Lactate dehydrogenase isoenzyme patterns upon chronic exposure to cigarette smoke: protective effect of bacoside A. Environ. Toxicol. Phar. 20, 345–350.
Anbarasi, K., Vani, G., Balakrishna, K., Shyamala Devi, C.S., 2006. Effect of bacoside A on brain antioxidant status in cigarette smoke exposed rats. Life Sci. 78, 1378–1384.
Ardite, E., Peinado, V.I., Rabinivich, R.A., Fernández-Checa, J.C., Roca, J., Barberà, J.A., 2006. Systemic effects of cigarette smoke exposure in the guinea pig. Respir. Med. 100, 1186–1194.
Arteel, G.E., Sies, H., 2001. The biochemistry of selenium and the glutathione system. Environ. Toxicol. Phar. 10, 153–158.
Avti, P.K., Kumar, S., Pathak, C.M., Vaiphei, K., Khanduja, K.L., 2006. Smokeless tobacco impairs the antioxidant defense in liver, lung, and kidney of rats. Toxicol. Sci. 89 (2), 547–553.
Barber, K., Mussin, E., Taylor, D.K., 1996. Fetal exposure to involuntary maternal smoking and childhood respiratory disease. Ann. Allerg. Asthma Immunol. 76, 427–430.
Baskaran, S., Lakshimi, S., Prasad, P.R., 1999. Effect of cigarette smoke on lipid peroxidation and antioxidant enzymes in albino rat. Indian J. Exp. Biol. 37, 1196–1200.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein–dye binding. Anal. Biochem. 72, 248–254.
Church, D.F., Pryor, W.A., 1985. Free radicals chemistry of cigarette smoke and its toxicological implications. Environ. Health Perspect. 64, 111–126.
Cross, C.E., O'Neill, C.A., Reznick, A.Z., Hu, M.L., Marcocci, L., Packer, L., Frei, B., 1993. Cigarette smoke oxidation of human plasma constituents. Ann. N.Y. Acad. Sci. 686, 72–90.
Cross, C.E., Valacchi, G., Schock, B., Wilson, M., Weber, S., Eiserich, J., Van der Vliet, A., 2002. Environmental oxidant pollutant effects on biologic systems. Am. J. Respir. Crit. Care 166, S44–S50.
Dejin-Karlsson, E., Hanson, B.S., Ostergren, P.O., Sjoberg, N.O., Marsal, K., 1998. Does passive smoking in early pregnancy increase the risk of small-for-gestational-age infants? Am. J. Public Health 88, 1523–1527.
El-Bayoumy, K., 2001. The protective role of selenium on genetic damage and on cancer. Mutat. Res. 475, 123–139.
Ellman, G.L., 1959. Tissue sulfhydryl groups. Arch. Biochem. 82, 70–77.
Folmer, V., Soares, J.C.M., Rocha, J.B.T., 2002. Oxidative stress in mice is dependent on the free glucose content of the diet. Int. J. Biochem. Cell. Biol. 34, 1279–1285.
Genbacev-Krtolica, O., 2005. Highlight for phenols, quinolines, indoles, benzene and 2-cyclopenten-1-ones are oviduct toxicants in cigarette smoke, by Prue Talbot, Karen Riveles, and Ryan Rosa: list of tobacco-smoke constituents that are harmful for reproduction grows-passive smokers may be at risk. Toxicol. Sci. 86 (1), 4–5.
Halliwell, B., Gutteridge, J.M.C. (Eds.), 2000. Free Radicals in Biology and Medicine, third ed. Oxford Science Publications, Oxford.
Hilbert, J., Mohsenin, V., 1996. Adaptation of lung antioxidants to cigarette smoking in humans. Chest 110, 916–920.
Ho, Y.S., 2002. Transgenic and knockout models for studying the role of lung antioxidant enzymes in defense against hyperoxia. Am. J. Respir. Crit. Care 166, S51–S56.
Hofhuis, W., de Jongste, J.C., Merkus, P.J., 2003. Adverse health effects of prenatal and postnatal tobacco smoke exposure on children. Arch. Dis. Child. 88, 1086–1090.
Husain, K., Scott, B.R., Reddy, S.K., Somani, S.M., 2001. Chronic ethanol and nicotine interaction on rat tissue antioxidant defense system. Alcohol 25, 89–97.
Jacques-Silva, M.C., Nogueira, C.W., Broch, L.C., Flores, E.M.M., Rocha, J.B.T., 2001. Diphenyl diselenide and ascorbic acid changes deposition of selenium and ascorbic acid in liver and brain of mice. Pharm. & Toxicol. 88, 119–125.
Kallner, A.B., Hartmann, D., Hornig, D.H., 1981. On the requirements of ascorbic acid in man: steady state turnover and body pool in smokers. Am. J. Clin. Nutr. 34, 1347–1355.
Klonoff-Cohen, H.S., Edelstein, S.L., Lefkowitz, E.S., 1995. The effect of passive smoking and tobacco exposure through breast milk on sudden infant death syndrome. JAMA 273, 795–798.
Meotti, F.C., Borges, V.C., Zeni, G., Rocha, J.B.T., Nogueira, C.W., 2003. Potential renal and hepatic toxicity of diphenyl diselenide, diphenyl diteluride and ebselen for rats and mice. Toxicol. Lett. 143, 9–16.
Meotti, F.C., Stangherlin, E.C., Zeni, G., Nogueira, C.W., Rocha, J.B.T., 2004. Protective role of aryl and alkyl diselenides on lipid peroxidation. Environ. Res. 94, 276–282.
Misra, H.P., Fridovich, I., 1972. The role of superoxide anion in the autoxidation of epinephrine and simple assay for superoxide dismutase. J. Biol. Chem. 247, 3170–3175.
Nogueira, C.W., Meotti, F.C., Curte, E., Pilissao, C., Zeni, G., Rocha, J.B.T., 2003. Investigations into the potential neurotoxicity induced by diselenides in mice and rats. Toxicology 183, 29–37.
Nogueira, C.W., Zeni, G., Rocha, J.B.T., 2004. Organoselenium and organotellurium compounds: toxicology and pharmacology. Chem. Rev. 104, 6255–6286.
Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.
Pande, M., Flora, S.J.S., 2002. Lead induced oxidative damage and its response to combined administration of α-lipoic acid and succimers in rats. Toxicology 177, 187–196.
Paulmier, C., 1986. Selenium reagents and intermediates. In: Organic Synthesis. Pergamon, Oxford.
Panda, K., Chattopadhyay, R., Ghish, M.K., Chattopadhyay, D.J., Chatterjee, I.B., 1999. Vitamin C prevents cigarette smoke-induced oxidative damage of proteins and increased proteolysis. Free Radic. Biol. Med. 27, 1064–1079.
Park, E.M., Park, Y.M., Gwak, Y.S., 1998. Oxidative damage in tissues of rats exposed to cigarette smoke. Free Radic. Biol. Med. 25, 79–86.
Pryor, W.A., Prier, D.G., Church, D.F., 1983. Electron spin resonance study of mainstream and side-stream cigarette smoke: nature of the free radicals in gas phase smoke and cigarette tar. Environ. Health Perspect. 47, 345–355.
Pryor, W.A., Stone, K., 1993. Oxidants in cigarette smoke. Ann. N.Y. Acad. Sci. 686, 12–28.
Rahman, I., Morrison, D., Donalson, K., MacNee, W., 1996. Systemic oxidative stress in asthma, COPD and smokers. Am. J. Respir. Crit. Care 154, 1055–1060.
Reddy, S., Finkelstein, E.I., Wong, P., Phung, A., Cross, C.E., Van der Vliet, A., 2002. Identification of glutathione modifications by cigarette smoke. Free Radic. Biol. Med. 33, 1490–1498.
Rossato, J.I., Ketzer, L.A., Centurião, F.B., Silva, S.J.N., Lüdtke, D.S., Zeni, G., Rubin, M.A., Rocha, J.B.T., 2002. Antioxidant proper-

ties of new chalcogenides against lipid peroxidation in rat brain. Neurochem. Res. 27, 297–303.

Sassa, S., 1982. Delta-aminolevulinic acid dehydratase assay. Enzyme 28, 133–145.

Schumacher, J.N., Green, C.R., Best, F.W., Newell, M.P., 1977. Smoke composition: an excessive investigation of the water-soluble portion of cigarette smoke. J. Agric. Food Chem. 25, 310–320.

Smith, K.R., Uyeminami, D.L., Kodavanti, U.P., Crapo, J.D., Chang, L.Y., Pinkerton, K.E., 2002. Inhibition of tobacco smoke-induced lung inflammation by a catalytic antioxidant. Free Radic. Biol. Med. 33, 1106–1114.

Soares, J.C.M., Folmer, V., Rocha, J.B.T., 2003. Influence of dietary selenium supplementation and exercise on thiol-containing enzymes in mice. Nutrition 19, 627–632.

Sobczak, A., Golka, D., Szoltysek-Boldys, I., 2004. The effects of tobacco smoke on plasma alpha- and gamma-tocopherol levels in passive and active cigarette smokers. Toxicol. Lett. 151, 429–437.

Tiwari, A.K., 2004. Antioxidants: new-generation therapeutic base for treatment of polygenic disorders. Curr. Sci. 86, 1092–1102.

Tomaki, M., Sugiura, H., Koarai, A., Komaki, Y., Akita, T., Matsumoto, T., Nakanishi, A., Ogawa, H., Hattori, T., Ichinose, M., 2006. Decreased expression of antioxidant enzymes and increased expression of chemokines in COPD lung. Pulm. Pharmacol. Ther., doi:10.1016/j.pupt.2006.06.006.

Tutka, P., Wielosz, M., Zatonski, W., 2002. Exposure to environmental tobacco smoke and children health. Int. J. Occup. Med. Environ. Health 15, 325–335.

US Department of Health and Human Services (DHHS), 1989. Reducing the health consequences of smoking: 25 years of progress. A report of the surgeon general. DHHS Publication No. 89-8411. CDC, Rockville, MD.

Wilson, S.R., Zucker, P.A., Huang, R.C., Spector, A., 1989. Development of synthetic compounds with glutathione peroxidase activity. J. Am. Chem. Soc. 111, 5936.

World Health Organization (WHO), 1992. Cadmium, Environmental Health Criteria, vol. 134. International Progress in Chemical Safety. WHO, Geneva.

World Health Organization (WHO), 1999. Tobacco free initiative. International consolation on environmental tobacco smoke and child health report. WHO, Geneva.

4.2. Artigo 2:

Efeito Antioxidante do Disseleneto de Difenila no Dano Induzido pela Fumaça em Ratos: Envolvimento da Glutationa

ANTIOXIDANT EFFECT OF DIPHENYL DISELENIDE ON OXIDATIVE DAMAGE INDUCED BY SMOKE IN RATS: INVOLVEMENT OF GLUTATHIONE

Cristiane Luchese, Eluza C. Stangherlin, Bibiana M. Gay, Cristina W. Nogueira

Ecotoxicology and Environmental Safety 72 (2009) 248-254

Ecotoxicology and Environmental Safety 72 (2009) 248–254

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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Antioxidant effect of diphenyl diselenide on oxidative damage induced by smoke in rats: Involvement of glutathione

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ARTICLE INFO

Article history:
Received 26 December 2007
Received in revised form
27 May 2008
Accepted 22 June 2008
Available online 3 August 2008

Keywords:
Cigarette smoking
Diphenyl diselenide
Selenium
Antioxidant
Oxidative stress
Glutathione

ABSTRACT

In the present study, the involvement of glutathione system in the restorative effect of diphenyl diselenide $(PhSe)_2$ on damage induced by cigarette smoke was investigated. Rat pups were progressively exposed to four, five, and six cigarettes for exposure periods of 15 min during their first, second, and third weeks of life. Thiobarbituric acid reactive species (TBARS) levels, components of the enzymatic antioxidant defenses (superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione *S*-transferase (GST) activities), and non-enzymatic antioxidant defenses (vitamin C and non-protein thiol (NPSH) levels) were examined in lungs of pups. The results demonstrated an increase in lipid peroxidation and the alteration in non-enzymatic and enzymatic antioxidant defenses induced by cigarette smoke exposure in lung of pups. Administration of $(PhSe)_2$ (0.5 mg/kg) restored TBARS levels and antioxidant defenses in lungs of rat pups exposed to cigarette smoke. $(PhSe)_2$ treatment increased NPSH levels and GST activity *per se* in lungs of rat pups. Together these results indicate that $(PhSe)_2$ restored oxidative damage induced by cigarette smoke exposure in lungs of rat pups. The glutathione system is involved in antioxidant effect of this compound.

1. Introduction

Cigarette smoking has been implicated as a major risk factor in the development of pulmonary, cardio and cerebrovascular diseases, cancers, and several others (USDHHS, 1989). The American Heart Association has formally concluded that passive smoking is an important risk factor for diseases in both adults and children (Taylor et al., 1992; Hofhuis et al., 2003). In addition, children are more likely than adults to suffer health effects from cigarette smoke exposure (Ashley and Ferrence, 1998). Compared with adults, they have higher respiratory rates, their bronchial tubes are smaller and their immune systems are less developed (WHO, 1999; Tutka et al., 2002). Passive smoking is an onset risk or exacerbation factor for various children's diseases such as bronchial asthma, lower respiratory illness, middle ear disease, and sudden infant death syndrome (Hofhuis et al., 2003).

Cigarette smoke is a complex mixture of over 4700 identified constituents (Rahman et al., 1996; Genbacev-Krtolica, 2005) that include numerous reactive substances such as a large quantity of reactive aldehydes (Park et al., 1998), free radical species (Pryor and Stone, 1993), and diverse metals (WHO, 1992). One pathway that may contribute to the unwanted health effects of cigarette smoking is exposure to oxidative stress (Alberg et al., 2000). Oxidative stress caused by cigarette smoke occurs due to the direct effects of the radicals present in smoke (Alberg, 2002). The presence and production of free radicals and other reactive oxygen and nitrogen species (ROS and NOS, respectively) from cigarette smoke may be the contributory factors to smoking-related diseases (Church and Pryor, 1985). They are capable of initiating or promoting oxidative damage (Schumacher et al., 1977; Pryor et al., 1983; Cross et al., 1993; Panda et al., 1999).

The oxidative effects of ROS are controlled by non-enzymatic antioxidant defenses, such as vitamins C, E, and non-protein thiol (NPSH), and also by enzymatic antioxidant defenses, such as scavenger enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) (Halliwell and Gutteridge, 2007). Accordingly, some authors have reported a protective role of antioxidants in modifying the major diseases related to cigarette smoking (Tiwari, 2004; Anbarasi et al., 2005).

The concept that selenium-containing molecules may be better nucleophiles (and therefore antioxidants) than classical antioxidants, has led to design synthetic organoselenium compounds (Arteel and Sies, 2001). Diphenyl diselenide, $(PhSe)_2$, retards the lipid peroxidation induced by a variety of oxidants (Rossato et al., 2002; Nogueira et al., 2004) and presents higher thiol peroxidase activity than other diselenides (Wilson et al., 1989; Meotti et al., 2004).

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doi:10.1016/j.ecoenv.2008.06.003

Previously, we reported that passive cigarette smoke exposure induced oxidative stress in lung of rat pups not only by increasing lipid peroxidation but also by reducing non-enzymatic antioxidant status (NPSH and ascorbic acid levels) (Luchese et al., 2007). Since the deleterious effects caused by smoke exposure were discrete, in the currently study we studied the effect of the progressive passive cigarette smoke exposure, in which animals were exposed to a higher number of cigarettes when compared with the previous one (Luchese et al., 2007).

In the present study, the involvement of glutathione system in the restorative effect of $(PhSe)_2$ on oxidative damage induced by progressive cigarette smoke exposure in lung of rat pups was evaluated.

2. Materials and methods

2.1. Chemicals

GR from baker's yeast, β-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH), and oxidized glutathione (GSSG) were purchased from Sigma (St. Louis, MO, USA). 1-Chloro-2,4-dinitrobenzene (CDNB) was purchased from Aldrich Chemical Co. (USA). $(PhSe)_2$ was prepared in our laboratory according to the method described in the literature (Paulmier, 1986).

Analysis of the ^1H NMR and ^{13}C NMR spectrum showed that $(PhSe)_2$ obtained presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of compound (99.9%) was determined by GC/HPLC. This drug was dissolved in canola oil, which was obtained from a standard commercial supplier. All other chemicals were of analytical grade and obtained from standard commercial suppliers. Commercial reference cigarettes, containing 0.8 mg nicotine and 0.9 mg tar, purchased from municipal stores were used. The cigarettes were of the same type for all cigarette smoke exposures.

2.2. Animals

Virgin female Wistar rats (180–240 g) from our own breeding colony were used. The animals were kept on a 12 h light/dark cycle, at a room temperature of 22 ± 2 °C, with free access to food and water. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, the Federal University of Santa Maria, Brazil. Sexually naive female rats were mated with male previously tested as fertile (three females and one male in each cage). The onset of pregnancy was confirmed by the presence of sperm in vaginal smears (day 0 of pregnancy) and pregnant dams were immediately housed in individual cages.

At birth, all litters were culled to eight pups. Whenever possible, only male rats were kept within the litter. Female rats were kept just to maintain equal litter sizes. Each litter presented pups (female and male) representative from each group of treatment.

2.3. Cigarette smoke exposure

Rat pups were divided into four groups (Table 1) of eight animals each, initially weighting 5–6 g. Pups belonging to groups 2 and 4 received daily oral administration of $(PhSe)_2$ (0.5 mg/kg, 2 μl of volume). Animals of groups 1 and 3 received oral application of canola oil (2 μl of volume). The oral application was given using an appropriated micropipette. After the treatment (around 5–10 min) rat pups of groups 3 and 4 were exposed to cigarette smoke.

Rat pups were placed in a whole body smoke exposure chamber (40 cm long, 30 cm wide, and 25 cm high), inside an exhaustion chapel, for progressive cigarette smoke exposure. The cigarettes were coupled to a small box, which was placed in the center of the exposure chamber. This apparatus produces sidestream smoke, which simulates environmental tobacco smoke or passive smoke exposure. The chamber has holes that facilitate the exit of carbon monoxide and entry of oxygen. The duration of each exposure period was 15 min, the time enough to cigarettes burn completely. Pups were progressively exposed to four, five, and six cigarettes during their first, second and third weeks of life. This protocol of exposure is defined as acutely toxic in view of the whole period of exposure, i.e., 3 weeks.

Table 1
Protocol of rat pups exposure

Groups	Treatments
Group 1	Canola oil (oral)+without cigarette smoke exposure
Group 2	Diphenyl diselenide (oral)+without cigarette smoke exposure
Group 3	Canola oil (oral)+cigarette smoke exposure
Group 4	Diphenyl diselenide (oral)+cigarette smoke exposure

Animals were exposed to cigarette smoke during 3 weeks (lactation period), 7 times/week.

Particulate levels/CO/PAHs in the chamber and cotinine in rat pups were not determined in view of experimental limitations. In addition, some authors have reported that the levels of cotinine are not directly related to the smoke exposure (Ebbert et al., 2004; Czekaj et al., 2002). Although the parameters described above are important to evaluate how much smoke the animals were exposed, it is not the objective of this study to extrapolate the data to human smokers.

To verify the validity of the cigarette smoke exposure protocol, carboxyhemoglobin (HbCO), pCO_2 and pO_2 levels were measured in arterial blood, collected of carotid artery, of rat pups. The levels of HbCO, pCO_2, and pO_2 were 3.5 ± 0.5%, 36.6 ± 5.6 and 68.0 ± 15.0 mmHg, respectively. The levels of HbCO and pCO_2 were 7.0- and 1.0-fold higher in rat pups exposed to cigarette smoke than the levels found in the control group. pO_2 of animals exposed to cigarette smoke was 1.4-fold lower than the level determined in the control group. These data confirm that animals were actually exposed to cigarette smoke.

At the end of experimental exposure period (3 weeks), rat pups were killed by decapitation and lungs were immediately excised. The choice of 3 weeks for the exposure period was based on studies which reported that the lung structure is complete around day 21 after birth (Yokoyama, 1983; Burri, 1974, 2006; Tschanz et al., 2003). The samples of lungs were homogenized in 50 mM Tris–HCl, pH 7.5 (1/5, w/v) and centrifuged at 2400g for 15 min at 4 °C. The low-speed supernatants (S1) were separated and used for biochemical assays.

2.4. Body weight gain

The body weight gain of pups was monitored for the whole course of the experiment. The body weight gain was calculated according to the following formula: final body weight–initial body weight.

2.5. Non-protein thiols (NPSH) determination

NPSH levels were determined by the method of Ellman (1959). To determine NPSH, S1 was mixed (1:1) with 10% trichloroacetic acid. After the centrifugation, the protein pellet was discarded and free –SH groups were determined in the clear supernatant. An aliquot of supernatant was added in 1 M potassium phosphate buffer pH 7.4 and 10 mM DTNB. The color reaction was measured at 412 nm. NPSH levels were expressed as μmol NPSH/g tissue.

2.6. Glutathione peroxidase (GPx) activity

GPx activity in S1 was assayed spectrophotometrically by the method of Wendel (1981), through the GSH/NADPH/GR system, by the dismutation of H_2O_2 at 340 nm. S1 was added in GSH/NADPH/GR system and the enzymatic reaction was initiated by adding H_2O_2. In this assay, the enzyme activity is indirectly measured by means of NADPH decay. H_2O_2 is decomposed, generating GSSG from GSH. GSSG is regenerated back to GSH by GR presents in the assay media at the expenses of NADPH. The enzymatic activity was expressed as nmol NADPH/min/mg protein.

2.7. GR activity

GR activity in S1 was determined as described by Calberg and Mannervik (1985). In this assay, GSSG is reduced by GR at the expense of NADPH consumption, which is followed at 340 nm. GR activity is proportional to NADPH decay. The enzymatic activity was expressed as nmol NADPH/min/mg protein.

2.8. Glutathione S-transferase (GST) activity

GST activity was assayed spectrophotometrically at 340 nm by the method of Habig et al. (1974). The reaction mixture contained an aliquot of S1, 0.1 M potassium phosphate buffer pH 7.4, 100 mM GSH and 100 mM CDNB, which was used as substrate. The enzymatic activity was expressed as nmol CDNB conjugated/min/mg protein.

2.9. CAT activity

CAT activity in S1 was assayed spectrophotometrically by the method of Aebi (1984), which involves monitoring the disappearance of H_2O_2 in the presence of S1 at 240 nm. An aliquot of S1 was added in 50 mM potassium phosphate buffer pH 7.0 and the enzymatic reaction was initiated by adding H_2O_2. One unit of enzyme was defined as the amount of enzyme required for monitoring the

disappearance of H_2O_2. The enzymatic activity was expressed as Units (U)/mg protein (1 U decomposes 1 μmol H_2O_2/min at pH 7 at 25 °C).

2.10. SOD activity

SOD activity was assayed spectrophotometrically as described by Misra and Fridovich (1972). This method is based on the capacity of SOD in inhibiting autoxidation of epinephrine to epinechrome. The color reaction was measured at 480 nm. At the test day, S1 was diluted 1:10 (v/v) for determination of SOD activity. Aliquots of S1 were added in a 50 mM glycine buffer pH 10.3 and the enzymatic reaction was initiated by adding epinephrine. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50% at 26 °C. The enzymatic activity was expressed as U/mg protein.

2.11. Thiobarbituric acid reactive species (TBARS) levels

TBARS levels, a measure of lipid peroxidation, were determined as described by Ohkawa et al. (1979). An aliquot of S1 was incubated with 0.8% thiobarbituric acid (TBA), acetic acid buffer pH 3.4 and 8.1% sodium dodecil sulphate at 95 °C for 2 h. The color reaction was measured at 532 nm. TBARS levels were expressed as nmol MDA (malondialdehyde)/mg protein.

This method has been subject to pervasive criticism due to the existence of a large range of non-lipid oxidation products in this system that also react with TBA to form colored species that can interfere with this assay (Halliwell and Gutteridge, 2007).

2.12. Ascorbic acid determination

Ascorbic acid determination was performed as described by Jacques-Silva et al. (2001). S1 was precipitated in 10 volumes of a cold 4% trichloroacetic acid solution. An aliquot of the sample in a final volume of 1 ml of the solution was incubated for 3 h at 38 °C then 65% H_2SO_4 (v/v) was added to the medium. The reaction product was determined using color reagent containing 4.5 mg/ml dinitrophenyl hydrazine and $CuSO_4$ (0.075 mg/ml). The color reaction was measured spectrophotometrically at 520 nm. Ascorbic acid content was expressed as μg ascorbic acid/g tissue.

2.13. Protein determination

Protein concentration was measured by the method of Bradford (1976), using bovine serum albumin as the standard.

2.14. Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed using a two-way ANOVA followed by Duncan's test. Main effects are presented only when the higher second-order interaction was non-significant. Values of $p<0.05$ were considered statistically significant.

3. Results

3.1. Body weight gain

There was no change in the body weight gain of pups in none of experimental groups during the period of exposure. The body weight gain of animals were 38.6 ± 4.3 (control group), 40.1 ± 3.2 ($(PhSe)_2$ group), 38.2 ± 4.0 (smoke group), and 39.0 ± 3.6 g ($(PhSe)_2$+smoke group).

3.2. NPSH levels

Two-way ANOVA of NPSH levels yielded a significant main effect of cigarette smoke exposure and $(PhSe)_2$ ($F_{1,47} = 4.826$ and $F_{1,47} = 49.106$, $p<0.05$, respectively). Post-hoc comparisons demonstrated that $(PhSe)_2$ restored NPSH levels reduced by cigarette smoke exposure in lung of rat pups. Treatment with $(PhSe)_2$ increased *per se* NPSH levels (22%) in lung of pups (Fig. 1).

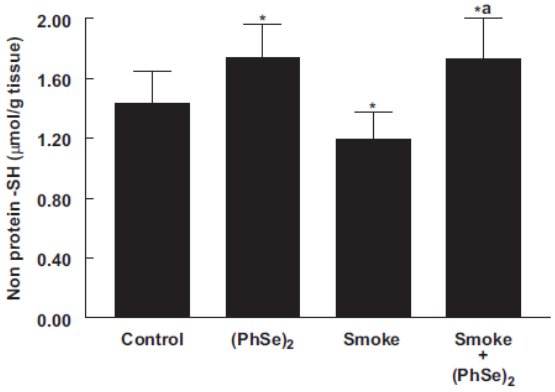

Fig. 1. Effect of $(PhSe)_2$ on NPSH levels in lungs of rat pups exposed to cigarette smoke. Data are reported as mean ± SD of seven animals per group. (*) Denoted $p<0.05$ as compared with the control group (two-way ANOVA/Duncan). (a) Denoted $p<0.05$ as compared with the smoke group (two-way ANOVA/Duncan).

3.3. GPx activity

Two-way ANOVA of GPx activity yielded a significant main effect of cigarette smoke exposure ($F_{1,15} = 7.202$, $p<0.05$). *Post-hoc* comparisons demonstrated that $(PhSe)_2$ did not restore GPx activity increased by cigarette smoke exposure in lung of rat pups (Fig. 2a).

3.4. GR activity

Two-way ANOVA of GR activity demonstrated a significant cigarette smoke exposure × $(PhSe)_2$ interaction ($F_{1,20} = 43.423$, $p<0.05$). *Post-hoc* comparisons demonstrated that $(PhSe)_2$ restored inhibition in GR activity caused by cigarette smoke exposure (34.2% of inhibition) in lung of rat pups (Fig. 2b).

3.5. GST activity

Two-way ANOVA of GST activity demonstrated a significant cigarette smoke exposure × $(PhSe)_2$ interaction ($F_{1,29} = 5.562$, $p<0.05$). *Post-hoc* comparisons showed that $(PhSe)_2$ restored inhibition of GST activity (26% of inhibition) in lung of rat pups. $(PhSe)_2$ increased *per se* GST activity (29.6%) in lung of rat pups (Fig. 2c).

3.6. CAT activity

Two-way ANOVA of CAT activity demonstrated a significant cigarette smoke exposure × $(PhSe)_2$ interaction ($F_{1,44} = 5.774$, $p<0.05$). *Post-hoc* comparisons demonstrated that $(PhSe)_2$ restored inhibition of CAT activity (19% of inhibition) induced by cigarette smoke exposure in lung of rat pups (Fig. 3).

3.7. SOD activity

Two-way ANOVA of SOD activity revealed a significant cigarette smoke exposure × $(PhSe)_2$ interaction ($F_{1,24} = 10.895$, $p<0.05$). *Post-hoc* comparisons showed that $(PhSe)_2$ restored SOD activity inhibited (32% of inhibition) by cigarette smoke exposure in lungs of rat pups (Fig. 4).

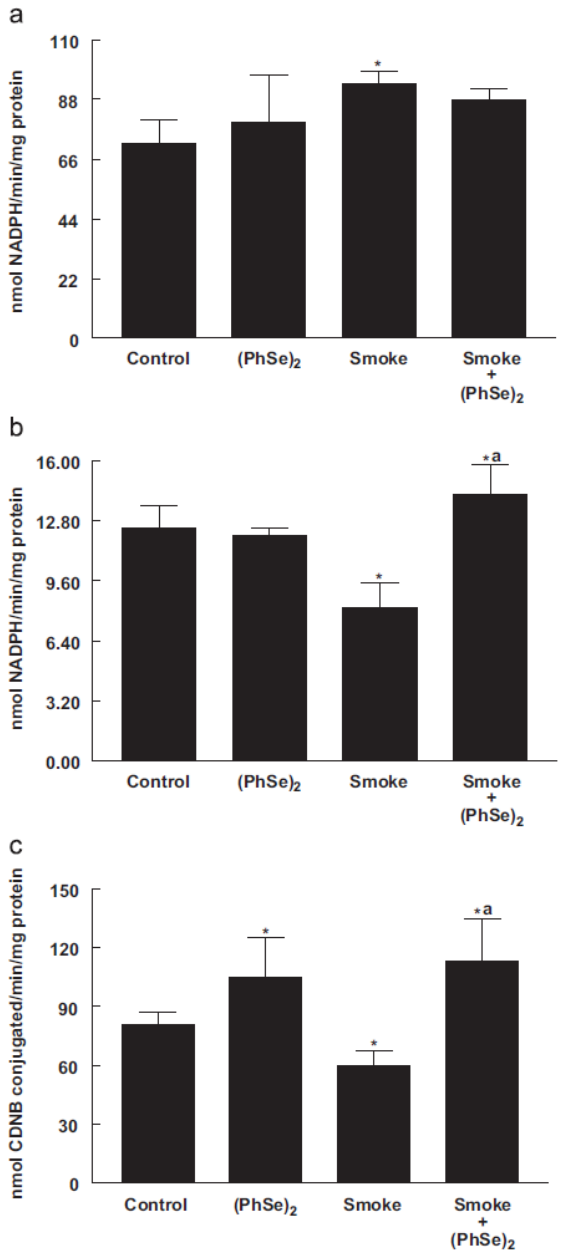

Fig. 2. Effect of $(PhSe)_2$ on GPx (2a), GR (2b), and GST (2c) activities in lungs of rat pups exposed to cigarette smoke. Data are reported as mean ± SD of seven animals per group. (*) Denoted $p<0.05$ as compared with the control group (two-way ANOVA/Duncan). (a) Denoted $p<0.05$ as compared with the smoke group (two-way ANOVA/Duncan).

3.8. Lipid peroxidation

Two-way ANOVA of TBARS levels yielded a significant cigarette smoke exposure × $(PhSe)_2$ interaction ($F_{1,42}=4.553$, $p<0.05$). *Post-hoc* comparisons indicated that $(PhSe)_2$ restored TBARS levels

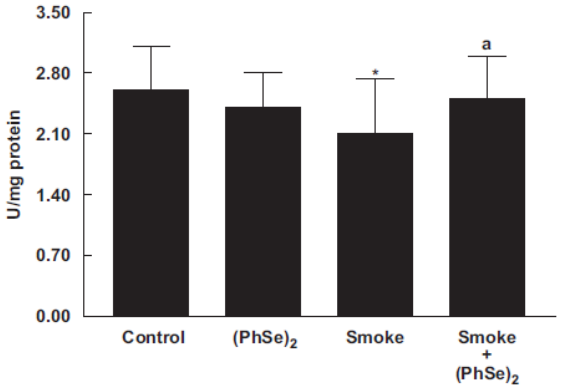

Fig. 3. Effect of $(PhSe)_2$ on CAT activity in lungs of rat pups exposed to cigarette smoke. Data are reported as mean ± SD of seven animals per group. (*) Denoted $p<0.05$ as compared with the control group (two-way ANOVA/Duncan). (a) Denoted $p<0.05$ as compared with the smoke group (two-way ANOVA/Duncan).

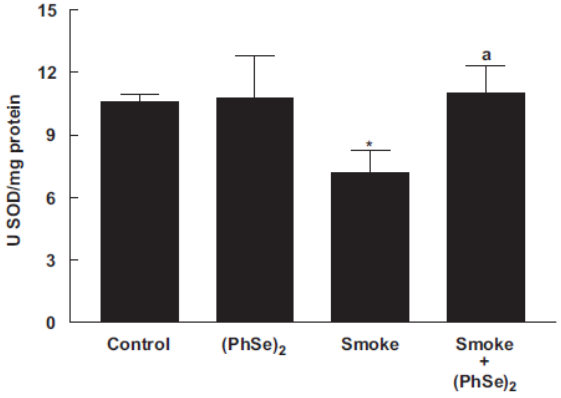

Fig. 4. Effect of $(PhSe)_2$ on SOD activity in lungs of rat pups exposed to cigarette smoke. Data are reported as mean ± SD of seven animals per group. (*) Denoted $p<0.05$ as compared with the control group (two-way ANOVA/Duncan). (a) Denoted $p<0.05$ as compared with the smoke group (two-way ANOVA/Duncan).

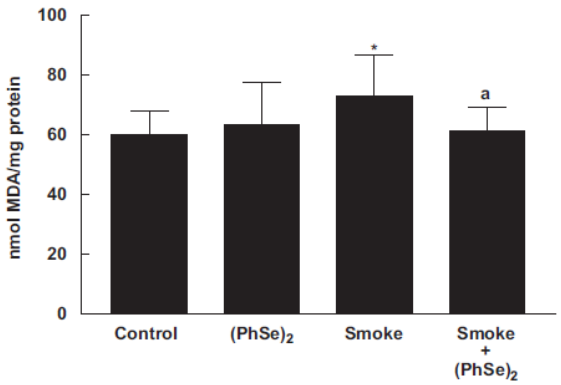

Fig. 5. Effect of $(PhSe)_2$ on TBARS levels in lungs of rat pups exposed to cigarette smoke. Data are reported as mean ± SD of seven animals per group. (*) Denoted $p<0.05$ as compared with the control group (two-way ANOVA/Duncan). (a) Denoted $p<0.05$ as compared with the smoke group (two-way ANOVA/Duncan).

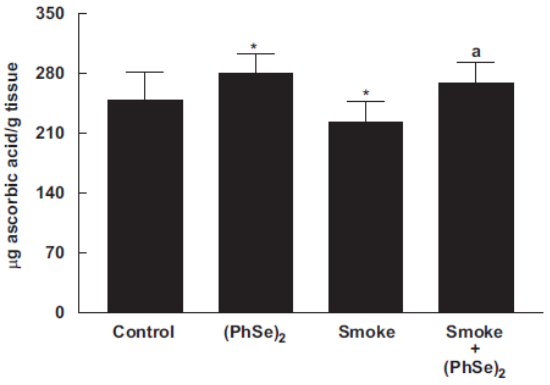

Fig. 6. Effect of $(PhSe)_2$ on ascorbic acid levels in lungs of rat pups exposed to cigarette smoke. Data are reported as mean ± SD of seven animals per group. (*) Denoted $p<0.05$ as compared with the control group (two-way ANOVA/Duncan). (a) Denoted $p<0.05$ as compared with the smoke group (two-way ANOVA/Duncan).

increased (1.21-fold) by cigarette smoke exposure in lung of rat pups (Fig. 5).

3.9. Ascorbic acid determination

Two-way ANOVA of ascorbic acid content yielded a significant main effect of cigarette smoke exposure and $(PhSe)_2$ ($F_{1,41}=5.655$ and $F_{1,41}=24.481$, $p<0.05$, respectively). *Post-hoc* comparisons showed that $(PhSe)_2$ ameliorated ascorbic acid levels reduced by cigarette smoke exposure in lungs of rat pups. $(PhSe)_2$ significantly increased *per se* ascorbic acid levels in lungs of rat pups (13%) (Fig. 6).

4. Discussion

The lung is the primary organ that becomes exposed to cigarette smoke and is highly susceptible to free radical generation. The lung has a rich network of antioxidant defenses to protect itself from free radical injury (Foronjy and D'Armiento, 2006). A complex antioxidant defense system of the respiratory tract consists of antioxidant enzymes (GPx, GR, SOD, and CAT) and numerous non-enzymatic antioxidants (NPSH and ascorbic acid), which are the line of defense against oxidants (Van der Vliet and Cross, 2000).

In the human scenario, offspring would likely be exposed to environmental tobacco smoke or passive smoke exposure during lactation as well as later in postnatal development. The main objective of this research was to study the effect of progressive cigarette smoke exposure for periods of 15 min during the first, second and third weeks of rat life. As a consequence, we studied the effect of cigarette smoke exposure in pups exposed to cigarette smoke and not their mothers. Thus, the information about what smoke components can pass into the milk that might be associated with observed effects is not relevant.

We found that pups exposed to 4–6 cigarettes during their first, second and third weeks of life presented oxidative damage in lung, which was verified by the increase in lipid peroxidation and a marked reduction in non-enzymatic antioxidant parameters (NPSH and ascorbic acid levels). These data are in agreement with previous data published by us (Luchese et al., 2007), in which the rats were exposed to 1–3 cigarettes during the same period of exposure. The contribution of the currently study to the results given in the previous paper is a more pronounced oxidative damage supported by the following data: inhibition in SOD and CAT activities, inhibition in GST and GR activities, and an increase in GPx activity. In the current study, treatment with $(PhSe)_2$ restored oxidative damage induced by cigarette smoke exposure in lungs of rat pups, and the glutathione system seems to be involved in antioxidant effect of this compound.

NPSH is an intracellular antioxidant and in some body compartments, such as the epithelial lining fluid of the lung, is present in high concentrations (Wendel and Cikryt, 1980; Meister and Anderson, 1983; Cantin et al., 1987; Deneke and Fanburg, 1989; Cross et al., 1994; Moss et al., 2000). NPSH in the lung lining plays a critical role in protecting the lung from oxidative stress by detoxifying exogenous and endogenous toxicants, and quenching ROS (Heffner and Rapine, 1989; Rahman and MacNee, 1999). According to Rahman and MacNee (2000) in pulmonary tissue, NPSH has been implicated in various cellular events, such as inflammatory response, modulation of redox-regulated signal transduction, regulation of cell proliferation, remodeling of extracellular matrix, maintenance of surfactant and antiprotease screen, apoptosis, immune modulation and mitochondrial respiration. In the present study, the levels of NPSH were reduced in lungs of rat pups exposed to cigarette smoke. In agreement with these data, various experimental research studies (Anand et al., 1996; Baskaran et al., 1999; Reddy et al., 2002; Ardite et al., 2006; Luchese et al., 2007) have demonstrated that cigarette smoke exposure induced depletion of NPSH in lungs of rats. Therefore, this may in part reflect the participation of this antioxidant defense in the toxicological mechanisms by which cigarette smoke causes damage in lungs of rat pups. Evidence in the literature indicates that oxidative stress, provoked by tobacco, causes redox instability and activation of body protective mechanisms, which result in a decrease in the concentration of some antioxidants (Sobczak et al., 2004; Avti et al., 2006).

The current study demonstrated changes in GPx, GR, and GST activities caused by cigarette smoke exposure in lungs of rat pups. Rat pups progressively exposed to cigarette smoke showed inhibition of GR activity, an important enzyme for the maintenance of intracellular concentration of GSH. Calberg and Mannervik (1985) have reported that GSH is the major NPSH in lungs. On the basis of this finding, we may suggest that reduced availability of NPSH could cause the reduction of GR activity in lung of rat pups exposed to cigarette smoke.

Moreover our investigation revealed that cigarette smoke exposure stimulated GPx activity in lung of rat pups. Increased activity of GPx further suggests an adaptive mechanism to counteract increased lipid peroxidation and the inhibition of CAT activity. However, the mechanism by which the stimulation of GPx activity occurs is yet unknown. We can infer that cigarette smoke contains numerous compounds, including ROS, reductants, and bioactive unsaturated aldehydes (Repine et al., 1997; Kodama et al., 1997), which may contribute to enhance the enzyme activity in lung of rat pups. Accordingly, Comhair et al. (1999) have reported the increase in GPx activity in epithelial lining fluid of cigarette smoking individuals. GPx is a major enzymatic antioxidant defense in the lung tissue (Avisser et al., 1996; Repine et al., 1997; Comhair et al., 1999), which catalyzes the reaction of hydroperoxides with GSH to form GSSG and reduced product of the hydroperoxide (Meister and Anderson, 1983).

Another interesting finding of the present study is the inhibition of GST activity in lung of rat pups exposed to cigarette smoke. GST, also known as phase II enzymes, are widely distributed catalyzing and binding proteins which promote the conjugation of GSH with a variety of reactive electrophilic

compounds resulting to formation of substances which are easily excreted from the body (Chasseaund, 1979; Cervello et al., 1992). The inhibition of GST activity induced by cigarette smoke exposure may be associated with the depletion of GSH levels, determined indirectly by NPSH content in this study, and by an increase of lipid peroxidation levels. In this way, several authors have reported that GST is an antioxidant defense and serves to protect the tissues against oxidative stress (Mosialou and Morgenstern, 1989; Hayes and Pulford, 1995; Fiander and Schneider, 1999).

Our data also demonstrated that cigarette smoke exposure inhibited SOD and CAT activities in lung of rat pups. The inhibition in SOD and CAT activities may be due to the adaptive mechanism to counteract lipid peroxidation in lungs of rat pups. Another plausible explanation for the inhibition of SOD and CAT activities could be their inactivation by oxidants present in the cigarette smoke. The decrease of SOD and CAT activities might predispose the lung of rat pups to oxidative stress, because these enzymes catalyze the decomposition of ROS. Accordingly, the inhibition of CAT and SOD activities as a result of oxidative stress has been reported (Jenkins and Goldfrab, 1993; Anbarasi et al., 2006).

Ascorbic acid has been reported as the first strong reductant against cigarette smoke oxidants affording considerable protection to the cells (Kallner et al., 1981). Acid ascorbic levels were found reduced in lungs of rat pups exposed to cigarette smoke, suggesting the participation of this non-enzymatic antioxidant defense in the toxicological mechanisms by which cigarette smoke exposure caused damage in rats. The decrease observed in ascorbic acid and NPSH levels associated to the increase of lipid peroxidation levels clearly characterize oxidative stress induced by cigarette smoke exposure.

The present study demonstrated that treatment with $(PhSe)_2$ restored oxidative damage induced by cigarette smoke exposure in lungs of rat pups. The exact mechanism to explain the antioxidant action of $(PhSe)_2$ is not clear at the moment. However, $(PhSe)_2$ acts in different antioxidant lines of defense, which were clearly evidenced by the efficiency of this organoselenium compound in restoring the oxidative damage induced by cigarette smoke exposure. Furthermore, in the present study, $(PhSe)_2$ caused an increase *per se* in NPSH levels and in GST activity in lung of pups. These results reinforce the involvement of glutathione system in the antioxidant effect of $(PhSe)_2$. Evidence in the literature indicates that $(PhSe)_2$ is a potent antioxidant agent and presents higher thiol peroxidase activity than other diselenides (Wilson et al., 1989; Meotti et al., 2004).

Also relevant are the findings showing that rat pups exposed to $(PhSe)_2$ did not have differences in weight gain. Reduction in body weight gain is widely used as a sign of toxicity in animals (Stangherlin et al., 2006). Therefore, the lack of loss of weight in rat pups exposed to $(PhSe)_2$ reinforces the low toxicity of this compound. It is important to point out that the fact that rats did not have weight differences is independent of mechanism of action of $(PhSe)_2$.

The above findings show that the cigarette smoke exposure induced oxidative stress in lung of rat pups by augmenting lipid peroxidation and by altering the non-enzymatic and enzymatic antioxidant status. Furthermore, $(PhSe)_2$ treatment restored the oxidative damage in lung, suggesting that this compound could be a potent antioxidant therapy against toxicity induced by cigarette smoke in rat pups. The antioxidant effect of $(PhSe)_2$ is, at least in part, associated to the involvement of glutathione system. Although this study provides valuable guidance, our data should not be extrapolated to the population. Further studies are warranted to elucidate the precise mechanism of $(PhSe)_2$ antioxidant effect in lung of rat pups.

Acknowledgments

The financial support by FAPERGS, CAPES, and CNPq(PIBIC) is gratefully acknowledged. C. W.N. and B. M.G. are recipients of CNPq fellowship.

Funding sources: The financial support by FAPERGS, CAPES, and CNPq(PIBIC) is gratefully acknowledged.

The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, the Federal University of Santa Maria, Brazil.

References

Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 105, 121–126.
Alberg, A., 2002. The influence of cigarette smoking on circulating concentrations of antioxidant micronutrients. Toxicology 180, 121–137.
Alberg, A.J., Chen, J.C., Zhao, H., Hoffman, S.C., Comslock, G.W., Helzlsouer, K.J., 2000. Household exposure to passive cigarette smoking and serum micronutrient concentrations. Am. J. Clin. Nutr. 72, 1576–1582.
Anand, C.V., Anand, U., Agarwal, R., 1996. Anti-oxidant enzymes, gammaglutamyl transpeptidase and lipid peroxidation in kidney of rats exposed to cigarette smoke. Indian J. Exp. Biol. 34, 486–488.
Anbarasi, K., Sabitha, K.E., Devi, C.S.S., 2005. Lactate dehydrogenase isoenzyme patterns upon chronic exposure to cigarette smoke: protective effect of Bacoside A. Environ. Toxicol. Pharm. 20, 345–350.
Anbarasi, K., Vani, G., Balakrishna, K., Shyamala Devi, C.S.S., 2006. Effect of bacoside A on brain antioxidant status in cigarette smoke exposed rats. Life Sci. 78, 1378–1384.
Ardite, E., Peinado, V.I., Rabinivich, R.A., Fernández-Checa, J.C., Roca, J., Barberà, J.A., 2006. Systemic effects of cigarette smoke exposure in the guinea pig. Resp. Med. 100, 1186–1194.
Arteel, G.E., Sies, H., 2001. The biochemistry of selenium and the glutathione system. Environ. Toxicol. Pharmacol. 10, 153–158.
Ashley, M.J., Ferrence, R., 1998. Reducing children's exposure to environmental tobacco smoke in homes: issues and strategies. Tob. Control 7, 1–12.
Avisser, N., Finkelstein, J.N., Horowitz, S., Willey, J.C., Coy, E., Frampton, M.W., Watkins, R.H., Khullar, P., Xu, Y.L., Cohen, H.J., 1996. Extracellular glutathione peroxidase in human lung epithelial lining fluid and in lung cells. Am. J. Physiol. (Lung Cell Mol. Physiol.) 14, L173–L182.
Avti, P.K., Kumar, S., Pathak, C.M., Vaiphei, K., Khanduja, K.L., 2006. Smokeless tobacco impairs the antioxidant defense in liver, lung, and kidney of rats. Toxicol. Sci. 89, 547–553.
Baskaran, S., Lakshimi, S., Prasad, P.R., 1999. Effect of cigarette smoke on lipid peroxidation and antioxidant enzymes in albino rat. Indian J. Exp. Biol. 37, 1196–1200.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Anal. Biochem. 72, 248–254.
Burri, P.H., 1974. The postnatal growth of the rat lung. 3. Morphology. Anat. Rec. 180, 77–98.
Burri, P.H., 2006. Structural aspects of postnatal lung development-alveolar formation and growth. Biol. Neonate 89, 313–322.
Calberg, I., Mannervik, B., 1985. Glutathione reductase. Methods Enzymol. 113, 484–490.
Cantin, A.M., North, S.L., Hubbard, R.C., Crystal, R.G., 1987. Normal alveolar epithelial lining fluid contains high levels of glutathione. J. Appl. Physiol. 63, 152–157.
Cervello, I., Lafuente, A., Giralt, M., Mallol, J., 1992. Enhanced glutathione *S*-transferase (GST) activity in pregnant rats treated with benzo(a)pyrene. Placenta 13, 273–280.
Chasseaund, L.F., 1979. The role of glutathione and glutathione *S*-transferase in the chemicals carcinogens and other electrophilic agents. Adv. Cancer Res. 29, 175–274.
Church, D.F., Pryor, W.A., 1985. Free radicals chemistry of cigarette smoke and its toxicological implications. Environ. Health Perspect. 64, 111–126.
Comhair, S.A.A., Lewis, M.J., Bhathena, P.R., Hammel, J.P., Erzurum, S.C., 1999. Increased glutathione and glutathione peroxidase in lungs of individuals with chronic beryllium disease. Am. J. Respir. Crit. Care Med. 159, 1824–1829.
Cross, C.E., O'Neill, C.A., Reznick, A.Z., Hu, M.L., Marcocci, L., Packer, L., Frei, B., 1993. Cigarette smoke oxidation of human plasma constituents. Ann. N.Y. Acad. Sci. 686, 72–90.
Cross, C.E., Van der Vliet, A., O'Neill, C.A., Louie, S., Halliwell, B., 1994. Oxidants, antioxidants, and respiratory tract lining fluids. Environ. Health Perspect. 102 (Suppl. 10), 185–191.
Czekaj, P., Palasz, A., Lebda-Wyborny, T., Nowaczyk-Dura, G., Karczewska, W., Florek, E., Kaminski, M., 2002. Morphological changes in lungs, placenta, liver and kidneys of pregnant rats exposed to cigarette smoke. Int. Arch. Occup. Environ. Health 75, S27–S35.
Deneke, S.M., Fanburg, B.L., 1989. Regulation of cellular glutathione. Am. J. Physiol. Lung Cell Mol. Physiol. 257, 163–173.

Ebbert, J.O., Dalea, L.C., Nirellic, L.M., Schroederc, D.R., Moyerd, T.P., Hurta, R.D., 2004. Cotinine as a biomarker of systemic nicotine exposure in spit tobacco users. Addict. Behav. 29, 349–355.
Ellman, G.L., 1959. Tissue sulfhydryl groups. Arch. Biochem. 82, 70–77.
Fiander, H., Schneider, H., 1999. Compounds that induce isoforms of glutathione *S*-transferase with properties of a critical enzyme in defense against oxidative stress. Biochem. Biophys. Res. Commun. 262, 591–595.
Foronjy, R., D'Armiento, J., 2006. The effect of cigarette smoke-derived oxidants on the inflammatory response of the lung. Clin. Appl. Immunol. Rev. 6, 53–72.
Genbacev-Krtolica, O., 2005. Highlight for phenols, quinolines, indoles, benzene and 2 cyclopenten-1-ones are oviduct toxicants in cigarette smoke, by Prue Talbot, Karen Riveles, and Ryan Rosa: list of tobacco-smoke constituents that Are harmful for reproduction grows—passive smokers may be at risk. Toxicol. Sci. 86, 4–5.
Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione *S*-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.
Halliwell, B., Gutteridge, J.M.C., 2007. Free Radicals in Biology and Medicine, fourth ed. Clarendon Press, Oxford, Oxford Science Publications, UK.
Hayes, J.D., Pulford, D.J., 1995. The glutathione *S*-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit. Rev. Biochem. Mol. Biol. 30, 445–600.
Heffner, J.E., Rapine, J.E., 1989. Pulmonary strategies of antioxidant defense. Am. Rev. Respir. Dis. 140, 531–554.
Hofhuis, W., de Jongste, J.C., Merkus, P.J., 2003. Adverse health effects of prenatal and postnatal tobacco smoke exposure on children. Arch. Dis. Child 88, 1086–1090.
Jacques-Silva, M.C., Nogueira, C.W., Broch, L.C., Flores, E.M.M., Rocha, J.B.T., 2001. Diphenyl diselenide and ascorbic acid changes deposition of selenium and ascorbic acid in liver and brain of mice. Toxicol. Pharm. 88, 119–125.
Jenkins, R.R., Goldfrab, A., 1993. Introduction: oxidant stress, aging and exercise. Med. Sci. Sport. Exer. 25, 210–212.
Kallner, A.B., Hartmann, D., Hornig, D.H., 1981. On the requirements of ascorbic acid in man: steady state turnover and body pool in smokers. Am. J. Clin. Nutr. 34, 1347–1355.
Kodama, M., Kaneko, M., Aida, M., Inoue, F., Nakayama, T., Akimoto, H., 1997. Free radical chemistry of cigarette smoke and its implication in human cancer. Anticancer Res. 17, 433–437.
Luchese, C., Stangherlin, E.C., Ardais, A.P., Nogueira, C.W., Santos, F.W., 2007. Diphenyl diselenide prevents oxidative damage induced by cigarette smoke exposure in lung of rat pups. Toxicology 230, 189–196.
Meister, A., Anderson, M.E., 1983. Glutathione. Annu. Rev. Biol. Chem. 52, 711–760.
Meotti, F.C., Stangherlin, E.C., Zeni, G., Nogueira, C.W., Rocha, J.B.T., 2004. Protective role of aryl and alkyl diselenides on lipid peroxidation. Environ. Res. 94, 276–282.
Misra, H.P., Fridovich, I., 1972. The role of superoxide anion in the autoxidation of epinephrine and simple assay for superoxide dismutase. J. Biol. Chem. 247, 3170–3175.
Mosialou, E., Morgenstern, R., 1989. Activity of rat liver microsomal glutathione transferase toward products of lipid peroxidation and studies of the effect of inhibitors on glutathione-dependent protection against lipid peroxidation. Arch. Biochem. Biophys. 275, 289–294.
Moss, M., Guidot, D.M., Wong-Lambertina, M., Ten Hoor, T., Perez, R.L., Brown, L.A., 2000. The effects of chronic alcohol abuse on pulmonary glutathione homeostasis. Am. J. Respir. Crit. Care Med. 161, 414–419.
Nogueira, C.W., Zeni, G., Rocha, J.B.T., 2004. Organoselenium and organotellurium compounds: toxicology and pharmacology. Chem. Rev. 104, 6255–6286.
Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.
Panda, K., Chattopadhyay, R., Ghish, M.K., Chattopadhyay, D.J., Chatterjee, I.B., 1999. Vitamin C prevents cigarette smoke-induced oxidative damage of proteins and increased proteolysis. Free Radic. Biol. Med. 27, 1064–1079.
Park, E.M., Park, Y.M., Gwak, Y.S., 1998. Oxidative damage in tissues of rats exposed to cigarette smoke. Free Radic. Biol. Med. 25, 79–86.
Paulmier, C., 1986. Selenium Reagents and Intermediates. Organic Synthesis. Pergamon, Oxford.
Pryor, W.A., Stone, K., 1993. Oxidants in cigarette smoke. Ann. N.Y. Acad. Sci. 686, 12–28.
Pryor, W.A., Prier, D.G., Church, D.F., 1983. Electron spin resonance study of mainstream and side-stream cigarette smoke: nature of the free radicals in gas phase smoke and cigarette tar. Environ. Health Perspect. 47, 345–355.
Rahman, I., MacNee, W., 1999. Lung glutathione and oxidative stress: implications in cigarette smoke-induced airway disease. Am. J. Physiol. Lung Cell Mol. Physiol. 277, L1067–L1088.
Rahman, I., MacNee, W., 2000. Oxidative stress and regulation of glutathione in lung inflammation. Eur. Respir. J. 16, 534–554.
Rahman, I., Morrison, D., Donalson, K., MacNee, W., 1996. Systemic oxidative stress in asthma, COPD and smokers. Am. J. Resp. Crit. Care 154, 1055–1060.
Reddy, S., Finkelstein, E.I., Wong, P., Phung, A., Cross, C.E., Van der Vliet, A., 2002. Identification of glutathione modifications by cigarette smoke. Free Radic. Biol. Med. 33, 1490–1498.
Repine, J.E., Bast, A., Lankhorst, I., The Oxidative Stress Study Group, 1997. Oxidative stress in chronic obstructive pulmonary disease. Am. J. Resp. Crit. Care Med. 156, 341–357.
Rossato, J.I., Ktzer, L.A., Centurião, F.B., Silva, S.J.N., Ludtke, D.S., Zeni, G., Braga, A.L., Rubin, M.A., Rocha, J.B.T., 2002. Antioxidant propeties of new chalcogenides against lipid peroxidation in rat brain. Neurochem. Res. 27, 297–303.
Schumacher, J.N., Green, C.R., Best, F.W., Newell, M.P., 1977. Smoke composition: an excessive investigation of the water-soluble portion of cigarette smoke. J. Agric. Food Chem. 25, 310–320.
Sobczak, A., Golka, D., Szoltysek-Boldys, I., 2004. The effects of tobacco smoke on plasma alpha- and gamma-tocopherol levels in passive and active cigarette smokers. Toxicol. Lett. 151, 429–437.
Stangherlin, E.C., Favero, A.M., Weis, S.N., Zeni, G., Rocha, J.B.T., Nogueira, C.W., 2006. Assessment of reproductive toxicity in male rats following acute and sub-chronic exposures to diphenyl diselenide and diphenyl ditelluride. Food Chem. Toxicol. 44, 662–669.
Taylor, A.E., Johson, D.C., Kazemi, H., 1992. Environmental tobacco smoke and cardiovascular disease: a position paper from the Council on Cardiopulmonary and Critical Care, American Heart Association. Circulation 86, 1–4.
Tiwari, A.K., 2004. Antioxidants: new-generation therapeutic base for treatment of polygenic disorders. Curr. Sci. 86, 1092–1102.
Tschanz, S.A., Makanya, A.N., Haenni, B., Burri, P.H., 2003. Effects of neonatal high-dose short-term glucocorticoid treatment on the lung: a morphologic and morphometric study in the rat. Pediatr. Res. 53, 72–80.
Tutka, P., Wielosz, M., Zatonski, W., 2002. Exposure to environmental tobacco smoke and children health. Int. J. Occup. Med. Environ. Health 15, 325–335.
US Department of Health and Human Services (DHHS), 1989. Reducing the health consequences of smoking: 25 years of progress. A Report of the Surgeon General. DHHS Publication. CDC, Rockville, MD, vol. n0(CDC), pp. 89–8411.
Van der Vliet, A., Cross, C.E., 2000. Oxidants, nitrosants, and the lung. Am. J. Med. 109, 398–421.
Yokoyama, E., 1983. Ventilatory functions of normal rats of different ages. Comp. Biochem. Physiol. A 75, 77–80.
Wendel, A., 1981. Glutathione peroxidase. Methods Enzymol. 77, 325–333.
Wendel, A., Cikryt, P., 1980. The level and half-life of glutathione in human plasma. FEBS Lett. 120, 209–211.
Wilson, S.R., Zucker, P.A., Huang, R.C., Spector, A., 1989. Development of synthetic compounds with glutathione peroxidase activity. J. Am. Chem. Soc. 111, 5936.
World Health Organization (WHO), 1992. Cadmium, Environmental Health Criteria. International Progress in Chemical Safety, vol. 134, Geneva.
World Health Organization (WHO), 1999. Tobacco free initiative. International Consolation on Environmental Tobacco Smoke and Child Health Report, Geneva.

4.3. Artigo 3:

Exposição Passiva à Fumaça Induz um Dano Oxidativo no Cérebro de Ratos Jovens: Papel Protetor do Disseleneto de Difenila

PASSIVE SMOKE EXPOSURE INDUCES OXIDATIVE DAMAGE IN BRAINS OF RAT PUPS: PROTECTIVE ROLE OF DIPHENYL DISELENIDE

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Inhalation Toxicology 19 (2009) 1-7

Inhalation Toxicology, 2008; 00(00): 000–000

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RESEARCH ARTICLE

Passive smoke exposure induces oxidative damage in brains of rat pups: Protective role of diphenyl diselenide

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Abstract

The protective effect of diphenyl diselenide, $(PhSe)_2$, on oxidative stress induced by cigarette smoke exposure in brains of rat pups was evaluated. Animals were exposed to passive cigarette smoke (15 min/day) in two different experimental protocols: P1 (1, 2, and 3 cigarettes) and P2 (4, 5, and 6 cigarettes) for 3 weeks. Before each period of smoke exposure, animals received an oral administration of $(PhSe)_2$ (0.5 mg/kg). A number of toxicological parameters in the brain were examined, such as lipid peroxidation, δ-aminolevulinate dehydratase (δ-ALA-D) activity, and components of enzymatic (superoxide dismutase and catalase activities) and non-enzymatic antioxidant defenses (ascorbic acid and non-protein thiol levels). In P1, smoke exposure induced an inhibition of catalase activity and an increase of ascorbic acid levels. $(PhSe)_2$ treatment was able to protect catalase activity but not ascorbic acid levels. In P2, an augmentation of lipid peroxidation, a reduction of enzymatic and non-enzymatic antioxidant status, and an inhibition of δ-ALA-D activity caused by smoke exposure were found. $(PhSe)_2$ protected the brains of rat pups against oxidative damage induced by smoke exposure. The results are consistent with the antioxidant effect of $(PhSe)_2$ demonstrated by the reduction of oxidative changes caused by smoke exposure in the brains of pups.

Introduction

Partially reduced derivatives of oxygen, which are produced in aerobic organisms as part of normal physiological and metabolic processes, are toxic species, oxidizing numerous biomolecules, leading to tissue injury and cell death. These reactive oxygen species are continuously formed in the human body and removed by enzymatic and non-enzymatic antioxidant defense systems under normal conditions (Yu, 1994). When there is excessive addition of free radicals from exogenous sources to the endogenous production, the available tissue defense system becomes overwhelmed, resulting in oxidative damage to the tissues. Oxidative stress has been shown to be associated with age-related neurodegenerative disorders such as Alzheimer's disease (Coyle & Puttfarcken, 1993), Parkinson's disease (Olanow, 1990), and amyotrophic lateral sclerosis (Bowling et al., 1993).

A major exogenous source of free radicals is cigarette smoke. There is evidence that smoking is related to increased free radical production and antioxidant depletion. It has been demonstrated that a puff of cigarette smoke contains in the order of 10^{14} identified constituents (Church & Pryor, 1985). There is a preponderance of evidence showing a strong association between cigarette smoking and an alarming increase in the mortality rate from smoking-related diseases such as pulmonary, cardio-, and cerebrovascular diseases, cancers, and several others (US DHHS, 1989). Cigarette smoke is a complex mixture of over 4000 identified constituents (Genbacev-Krtolica, 2005) that include numerous reactive substances such as a large quantity of reactive aldehydes (Park et al., 1998), free radical species such as oxygen free radicals and nitrogen species (Pryor & Stone, 1993), and diverse metals such as cadmium (Cd^{2+}) (WHO, 1992). Several micronutrients and antioxidants have been experimentally proved to be effective protective agents against smoking induced oxidative stress (Dilsiz et al., 1999; Helen et al., 1999).

In this context, the concept that selenium-containing molecules may be better nucleophiles (and therefore antioxidants) than classical antioxidants has led to the design of

The financial support given by FAPERGS, CAPES, and CNPq is gratefully acknowledged. CWN is the recipient of CNPq fellowships.
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(Received 13 August 2008; revised 1 October 2008; accepted 3 October 2008)

ISSN 0895-8378 print/ISSN 1091-7691 online © 2008 Informa UK Ltd
DOI: 10.1080/08958370802526881

synthetic organoselenium compounds (Arteel & Sies, 2001). In addition, diphenyl diselenide, $(PhSe)_2$, retards the lipid peroxidation induced by a variety of oxidants (Rossato et al., 2002). Moreover, this organochalcogen has been shown to be a better antioxidant (Meotti et al., 2004) and less toxic to rodents than other selenium compounds (Nogueira et al., 2003; Meotti et al., 2003). Recently, we have suggested that $(PhSe)_2$ is a potent antioxidant, protecting against toxicity induced by passive smoking in the lung, an organ highly prone to oxidative stress (Luchese et al., 2007a).

Another aspect that must be pointed out here is that, although passive smoking is recognized as an important health problem in older subjects, there is a surprising shortage of information on the health consequences of passive smoking in infants, and relatively few studies have examined the effects of these substances on the developing brain in experimental animals.

Therefore, in this study, we investigated the possible protective effect of $(PhSe)_2$ on oxidative damage induced by cigarette smoke in the brains of rats at an early developmental stage.

Methods

Materials

$(PhSe)_2$ was synthesized according to the literature method (Paulmier, 1986). Analysis of the ^1H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra showed analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of $(PhSe)_2$ (99.9%) was determined by GC/HPLC (gas chromatography/high performance liquid chromatography). $(PhSe)_2$ is a solid compound, is very stable, and can be stored in the laboratory in simple flasks for a long time. $(PhSe)_2$ was diluted in canola oil, which was obtained from a standard commercial supplier. δ-Aminolevulinic acid (δ-ALA) and *p*-dimethylaminobenzaldehyde were purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade and obtained from standard commercial suppliers. Cigarettes of diverse commercial marks containing 0.8 mg of nicotine were used.

Animals

Litters of Wistar rats (0–21 days) from our own breeding colony were used. The animals were kept on a 12-h light/dark cycle, at a room temperature of 22 ± 2°C, with free access to food (Guabi, RS, Brazil) and water. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Brazil. At birth, all litters were culled to eight pups.

Cigarette Smoke Exposure

The experimental groups were:

- C: control, canola oil
- Se: animals exposed to $(PhSe)_2$
- S: animals exposed to passive smoking and canola oil
- S + Se: animals exposed to passive smoking and $(PhSe)_2$

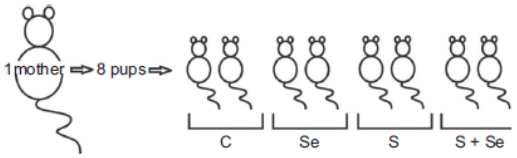

Figure 1. Distribution of litter for exposure to $(PhSe)_2$ and cigarette smoke. Animals were exposed to cigarette smoke during 3 weeks (lactation period), 7 times/week (P1: week 1, 1 cigarette/15 min, week 2, 2 cigarettes/15 min, and week 3, 3 cigarettes/15 min or P2: week 1, 4 cigarettes/15 min, week 2, 5 cigarettes/15 min, and week 3, 6 cigarettes/15 min). C, canola; Se, diphenyl diselenide; S, smoke.

Each experimental group consisted of at least ten suckling rats (male and female), initially weighting 5–6 g. The smoker animals were exposed to the smoke of commercial cigarettes during 3 weeks (lactation period/0–21 days of life). Each litter presented pups (female and male) that were represented in each experimental group (Figure 1).

For both protocols, rat pups were exposed to cigarette smoke in a whole body smoke exposure chamber for three consecutive weeks. The duration of each exposure was 15 min/day as follows:

Protocol 1 (P1). In the first, second, and third weeks of life, animals were concomitantly exposed to the smoke of one, two, and three cigarettes, respectively (week 1: 1 cigarette/15 min; week 2: 2 cigarettes/15 min; and week 3: 3 cigarettes/15 min).

Protocol 2 (P2). In the first, second, and third weeks of life, animals were concomitantly exposed to the smoke of four, five, and six cigarettes, respectively (week 1: 4 cigarettes/15 min; week 2: 5 cigarettes/15 min; and week 3: 6 cigarettes/15 min).

Before each period of smoke exposure, animals received an oral administration of $(PhSe)_2$ (0.5 mg/kg, 2 µl in volume) or canola oil (2 µl in volume). Control animals followed the same procedure except for the cigarette smoke exposure.

At the end of the experimental period (3 weeks), animals were killed by decapitation and brain tissues were immediately excised. The brain was rapidly homogenized in 50 mM Tris-HCl, pH 7.4 (1:5, w/v), and centrifuged at 2400 × *g* for 15 min at 4°C. The low-speed supernatants (S1) were separated and used for biochemical assays.

Body weight gain

The body weight gain of pups was monitored for the whole course of the experiment and calculated according to the following formula: final body weight – initial body weight.

Biochemical assays

Lipid peroxidation

Levels of thiobarbituric acid-reactive substances (TBARS) were determined as described by Ohkawa et al. (1979), and

this parameter was used as a measure of lipid peroxidation. An aliquot of S1 was incubated with 0.8% thiobarbituric acid (TBA), acetic acid buffer pH 3.4, and 8.1% sodium dodecyl sulfate (SDS) at 95°C for 2 hours. The color reaction was measured at 532 nm. TBARS levels were expressed as nmol MDA (malondialdehyde)/mg protein.

δ-Aminolevulinate dehydratase (δ-ALA-D) activity
δ-ALA-D activity is extremely sensitive to situations associated with oxidative stress; thus this enzyme has been used as an indicator of oxidative stress (Folmer et al., 2002; Nogueira et al., 2004; Luchese et al., 2007b). δ-ALA-D activity in the brain was assayed according to the method of Sassa (1982) by measuring the rate of product (porphobilinogen) formation, except that 1 M potassium phosphate buffer pH 6.8 and 12 mM ALA were used. An aliquot of S1 was incubated for 3 hours at 37°C. The reaction was linear in relation to protein and time of incubation. The reaction product was determined using modified Erlich's reagent at 555 nm. δ-ALA-D activity was expressed as nmol PBG (porphobilinogen)/mg protein/h.

Determination of non-protein thiols (NPSH)
NPSH in the brain were determined by the method of Ellman (1959). To determine NPSH in the brains of rat pups, S1 was mixed (1:1) with 10% trichloroacetic acid. After centrifugation, the protein pellet was discarded and free -SH groups were determined in the clear supernatant. An aliquot of clear supernatant was added to potassium phosphate buffer 1 M pH 7.4 and 10 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB). The color reaction was measured at 412 nm. NPSH levels were expressed as μmol NPSH/g tissue.

Ascorbic acid determination
Ascorbic acid determination was performed as described by Jacques-Silva et al. (2001). Protein (S1) was precipitated in 10 volumes of a cold 4% trichloroacetic acid solution. An aliquot of the clear supernatant in a final volume of 1 ml of solution was incubated for 3 hours at 38°C then H_2SO_4 65% (v/v) was added to the medium. The reaction product was determined using a color reagent containing 4.5 mg/ml dinitrophenyl hydrazine and $CuSO_4$ (0.075 mg/ml). The color reaction was measured spectrophotometrically at 520 nm. The ascorbic acid content was expressed as μg ascorbic acid/g tissue.

Superoxide dismutase (SOD) activity
SOD activity was assayed spectrophotometrically as described by Misra and Fridovich (1972). This method is based on the capacity of SOD to inhibit the autoxidation of adrenaline to adrenochrome. The color reaction was measured at 480 nm. On the test day, aliquots of S1 were added to a glycine buffer 50 mM pH 10.3. The enzymatic reaction was started by adding adrenaline. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of adrenaline autoxidation by 50% at 26°C. The enzymatic activity was expressed as units (U)/mg protein.

Catalase activity
The catalase activity was assayed spectrophotometrically using the method of Aebi (1984), which involves monitoring the disappearance of H_2O_2 in the presence of brain homogenate at 240 nm. An aliquot of S1 was added to 50 mM potassium phosphate buffer pH 7.0 and the enzymatic reaction was initiated by adding H_2O_2. The enzymatic activity was expressed in units (U)/mg protein (1 U decomposes 1 μmol H_2O_2/min at pH 7 at 25°C).

Protein Determination
Protein was measured by the Coomassie blue method according to Bradford (1976) using bovine serum albumin as standard. An aliquot of S1 diluted 1:10 (v/v) was added to Coomassie blue reactive. The color was measured spectrophotometrically at 595 nm.

Statistical analysis
Data are expressed as mean ± SD. Statistical analysis was performed using a two-way analysis of variance (ANOVA) followed by the Duncan test. Values of $p < 0.05$ were considered statistically significant.

Results

Body weight gain
There was no change in body weight gain of pups in none of the experimental groups during the period of exposure (data not shown).

Biochemical assays
Lipid peroxidation
In P1, two-way ANOVA of TBARS levels revealed that exposure to cigarette smoke did not alter lipid peroxidation in the brains of pups (Figure 2). In P2, two-way ANOVA of TBARS

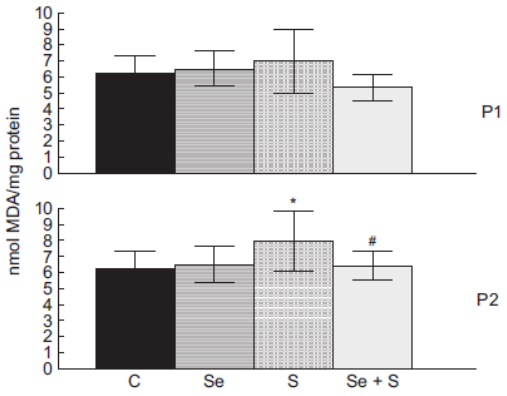

Figure 2. Effect of $(PhSe)_2$ on thiobarbituric acid-reactive substances (TBARS) levels in brains of pups after cigarette smoke exposure. Data are expressed as nmol MDA (malondialdehyde)/mg protein. Data are reported as mean ± SD for 10 animals per group (P1: 1, 2, 3 cigarettes/3 weeks and P2: 4, 5, 6 cigarettes/3 weeks). *$p < 0.05$ compared to control group (two-way ANOVA/Duncan). #$p < 0.05$ compared to smoke group (two-way ANOVA/Duncan).

levels yielded a significant cigarette smoke exposure–$(PhSe)_2$ interaction ($F_{1,43}=6.409$; $p<0.05$). Post-hoc comparisons demonstrated that cigarette smoke exposure increased lipid peroxidation in the brains of pups (1.3 times higher than in the control group) ($p<0.01$). $(PhSe)_2$ treatment protected against an enhancement of TBARS levels caused by cigarette smoke exposure in the brains of pups (Figure 2).

δ-ALA-D activity

In P1, two-way ANOVA of δ-ALA-D activity revealed a significant main effect of cigarette smoke exposure ($p<0.01$). Post-hoc comparisons showed that exposure to cigarette smoke did not alter the enzyme activity in the brains of pups (Figure 3). Two-way ANOVA of δ-ALA-D activity yielded a significant main effect of cigarette smoke exposure ($p<0.01$) in P2. Post-hoc comparisons demonstrated that cigarette smoke exposure inhibited enzyme activity (around 15% inhibition) in the brains of pups ($p<0.05$). $(PhSe)_2$ treatment partially protected against the inhibition of δ-ALA-D activity by cigarette smoke exposure in the brains of pups (Figure 3).

NPSH levels

In P1, two-way ANOVA revealed that NPSH levels in the brains of pups were not altered by exposure to cigarette smoke (Figure 4). In contrast to P1, two-way ANOVA of NPSH levels yielded a significant cigarette smoke exposure–$(PhSe)_2$ interaction ($F_{1,59}=5.349$; $p<0.05$) in P2. Post-hoc comparisons demonstrated that cigarette smoke exposure decreased NPSH levels (around 12%) in the brains of pups ($p<0.05$). $(PhSe)_2$ treatment ameliorated NPSH levels altered by smoke exposure in the brains of pups (Figure 4).

Ascorbic acid content

In P1, two-way ANOVA of ascorbic acid content yielded a significant main effect of cigarette smoke exposure ($p<0.00001$). Post-hoc comparisons demonstrated that exposure to cigarette smoke significantly increased ascorbic acid levels (around 13%) in the brains of pups ($p<0.01$). $(PhSe)_2$ treatment was not effective in preventing the augmentation of ascorbic acid levels in the brains of pups (Figure 5).

Conversely, in P2, cigarette smoke exposure decreased ascorbic acid levels (around 8%) in the brains of pups ($p<0.05$). $(PhSe)_2$ treatment partially prevented a decrease of ascorbic acid content in the brains of pups (Figure 5).

SOD activity

In P1, two-way ANOVA of SOD activity demonstrated that exposure to cigarette smoke did not alter the enzyme activity in the brains of pups (Figure 6).

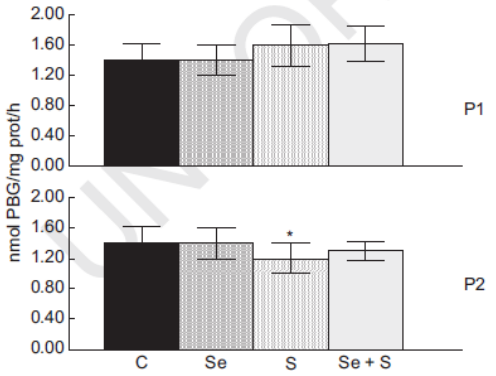

Figure 3. Effect of $(PhSe)_2$ on δ-aminolevulinate dehydratase (δ-ALA-D) activity in brains of pups after cigarette smoke exposure. Data are expressed as nmol PBG (porphobilinogen)/mg protein/h. Data are reported as mean ± SD for 10 animals per group (P1: 1, 2, 3 cigarettes/3 weeks and P2: 4, 5, 6 cigarettes/3 weeks). *$p<0.05$ compared to control group (two-way ANOVA/Duncan).

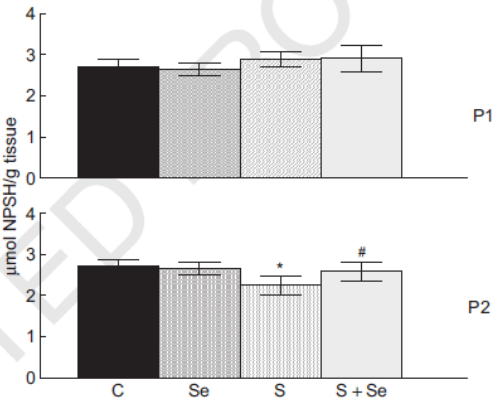

Figure 4. Effect of $(PhSe)_2$ on non-protein thiol groups (NPSH) content in brains of pups after cigarette smoke exposure. Data are expressed as µmol NPSH/g tissue. Data are reported as mean ± SD for 10 animals per group (P1: 1, 2, 3 cigarettes/3 weeks and P2: 4, 5, 6 cigarettes/3 weeks). *$p<0.05$ compared to control group (two-way ANOVA/Duncan). #$p<0.05$ compared to smoke group (two-way ANOVA/Duncan).

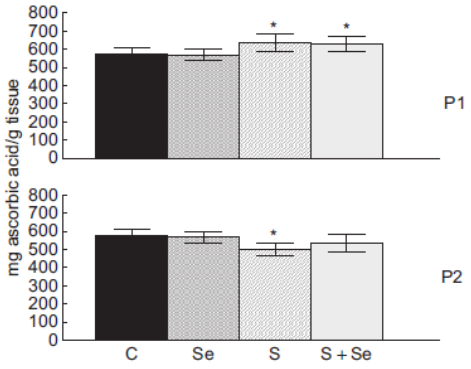

Figure 5. Effect of $(PhSe)_2$ on ascorbic acid levels in brains of pups after cigarette smoke exposure. Data are expressed as mg ascorbic acid/g tissue. Data are reported as mean ± SD for 10 animals per group (P1: 1, 2, 3 cigarettes/3 weeks and P2: 4, 5, 6 cigarettes/3 weeks). *$p<0.05$ compared to control group (two-way ANOVA/Duncan).

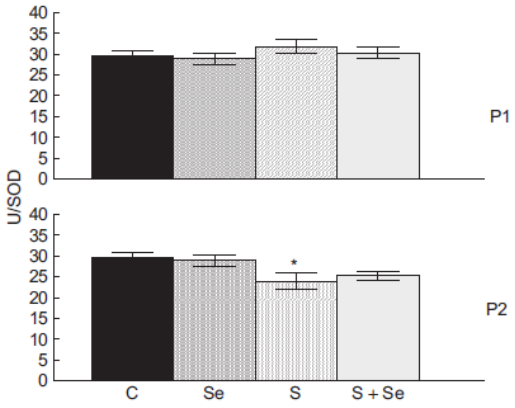

Figure 6. Effect of $(PhSe)_2$ on superoxide dismutase (SOD) in brains of pups after cigarette smoke exposure. Data are expressed as units (U)/mg protein. Data are reported as mean ± SD for 10 animals per group (P1: 1, 2, 3 cigarettes/3 weeks and P2: 4, 5, 6 cigarettes/3 weeks). *$p < 0.05$ compared to control group (two-way ANOVA/Duncan).

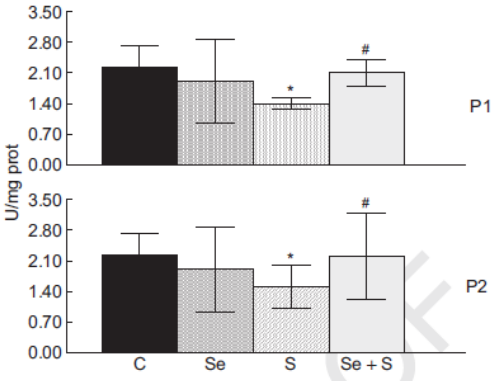

Figure 7. Effect of $(PhSe)_2$ on catalase activity in brains of pups after cigarette smoke exposure. Data are expressed as units (U)/mg protein. Data are reported as mean ± SEM for 10 animals per group (P1: 1, 2, 3 cigarettes/3 weeks and P2: 4, 5, 6 cigarettes/3 weeks). *$p < 0.05$ compared to control group (two-way ANOVA/Duncan). #$p < 0.05$ compared to smoke group (two-way ANOVA/Duncan).

Two-way ANOVA of SOD activity yielded a significant main effect of cigarette smoke exposure ($p < 0.0001$) in P2. Post-hoc comparisons demonstrated that cigarette smoke exposure inhibited enzyme activity (24% inhibition) in the brains of pups ($p < 0.05$). $(PhSe)_2$ treatment did not protect against the inhibition of SOD activity caused by cigarette smoke exposure in the brains of pups (Figure 6).

Catalase activity

In P1 and P2, two-way ANOVA of catalase activity yielded a significant cigarette smoke exposure–$(PhSe)_2$ interaction ($F_{1,29} = 4.519$ and $F_{1,40} = 5.393$, respectively; $p < 0.05$). In both P1 and P2, post-hoc comparisons demonstrated that cigarette smoke exposure inhibited enzyme activity (37% and 33%, respectively) in the brains of pups ($p < 0.05$). $(PhSe)_2$ treatment protected against the inhibition of catalase activity in the brains of pups in both experimental protocols (Figure 7).

Discussion and conclusions

The present study found that passive cigarette smoke exposure induces an increase of oxidative stress in the brains of rats in the early postnatal period, a period in which the brain is still in development. We observed an augmentation of lipid peroxidation, an inhibition of δ-ALA-D activity, and a reduction of enzymatic and non-enzymatic antioxidant status caused by cigarette smoke exposure. The data presented here are also consistent with the antioxidant effect of $(PhSe)_2$ demonstrated by the reduction of oxidative damage caused by cigarette smoke exposure.

The brain is extremely vulnerable to oxidative stress, in part because it is highly enriched with non-heme iron, which is catalytically involved in the production of oxygen free radicals. In addition, the brain contains a relatively high degree of polyunsaturated fatty acids that are particularly good substrates for peroxidation reactions (Halliwell & Gutteridge, 2000). Besides that, cigarette smoke contains numerous compounds and diverse metals (WHO, 1992), many of which are oxidants and pro-oxidants, capable of producing free radicals and enhancing the oxidative stress in vivo (Pryor & Stone, 1993).

This study comprised 3 weeks of exposure to cigarettes, an intermediate time, classified as sub-chronic. This time can reveal alterations observed in acute or chronic exposure. An increase in the activity of antioxidant enzymes has been demonstrated upon exposure to cigarette smoke for 4 weeks (Baskaran et al., 1999). Therefore, these conflicting data are probably due to differences in experimental protocols.

This study revealed that oxidative status in the brains of rat pups was only slightly altered by the P1 protocol. In fact, animals exposed to P1 had increased levels of ascorbic acid, which is the first strong reductant to be reported to react readily with cigarette smoke oxidants and afford considerable protection to the cells (Kallner et al., 1981). The increased levels of this antioxidant in P1 could be a result of an adaptive response to the noxious action of the cigarette constituents, independent of changes in lipid peroxidation. Accordingly, acute exposure to cigarette smoke enhances the production of this antioxidant defense as a result of the adaptive response, which consequently mitigates the damage caused by cigarette smoke (Hilbert & Mohsenin, 1996). An inhibition of catalase activity was also found after P1 exposure, which suggests inactivation of the enzyme by oxidants present in the cigarette smoke (Anbarasi et al., 2005). $(PhSe)_2$ treatment did not modify ascorbic acid levels but ameliorated the inhibition of catalase activity in the brains of pups exposed to cigarette smoke. Considering P1, it is difficult to explain why the levels of TBARS and NPSH, and the activities of δ-ALA-D and SOD, were not changed by cigarette smoke. The number of cigarettes used was perhaps responsible for the lack of alteration in these parameters.

Since protocol P1 used one, two, and three cigarettes over 3 weeks, it is possible that the number of cigarettes was insufficient to cause significant alterations.

Considering the P2 protocol, an increase of oxidative stress was found with the increase in number of cigarettes. Pups exposed to the P2 protocol presented oxidative damage in the brain, which was verified by the increase in lipid peroxidation, inhibition of δ-ALA-D activity, marked reduction in non-enzymatic antioxidant parameters (NPSH and ascorbic acid), and inhibition of SOD and catalase activities. These results are in accordance with previous data demonstrating that oxidative stress caused by cigarette smoke exposure causes a decrease in the concentration of some antioxidants (Sobczak et al., 2004; Avti et al., 2006).

In the P2 protocol, the levels of NPSH and ascorbic acid were reduced, and the activity of SOD and catalase was inhibited in the brains of pups. This effect could be associated with the elevation in lipid peroxidation levels in the brains of the pups. In addition, the ascorbic acid content was decreased in this tissue. The consumption of ascorbic acid by cigarette smoke exposure would explain why plasma ascorbic acid levels of chronic smokers are markedly lower than those of non-smokers (Brook & Grimshaw, 1968; Horning & Glatthaar, 1985). Low blood levels of ascorbic acid are characteristic, and can be restored following smoking cessation (Polidori et al., 2003). Cigarette smoke also damages low molecular weight thiols (O'Neill et al., 1994), and smoking is also associated with decreased plasma NPSH levels (Richards et al., 1996). Accordingly, various experimental studies have demonstrated that exposure to cigarette smoke induces a depletion of NPSH in several tissues (Anand et al., 1996; Baskaran et al., 1999; Reddy et al., 2002). In fact, the depletion of ascorbic acid and NPSH content is directly associated with the elevation of lipid peroxidation, which can be attributed to its protection against the effects of ROS generated by smoke. Thus, the changes observed in non-enzymatic antioxidant defenses (NPSH and ascorbic acid) after cigarette smoke exposure could be due to the adaptative response of cerebral tissue. Moreover, this may in part reflect the participation of these antioxidant defenses in toxicological mechanisms by which cigarette smoke exposure causes damage in the brains of pups.

Cigarette smoke exposure caused a decrease of SOD and catalase activities in the brains of pups. Probably, the inhibition of enzyme activities resulted from their inactivation by oxidants present in the cigarette smoke. In this way, we can suggest that the accumulation of superoxide anion radicals caused by cigarette smoke exposure in the brains of rat pups leads to the inhibition of catalase activity. The inhibition of SOD and catalase activities might predispose the brains of rat pups to an increase in oxidative damage, because these enzymes catalyze the decomposition of ROS.

δ-ALA-D activity was significantly inhibited by cigarette smoke in the brains of rat pups exposed to the P2 protocol. δ-ALA-D is a sulfhydryl-containing enzyme (Gibson et al., 1955) inhibited by numerous metals (Rocha et al., 1995; Rodrigues et al., 1996; Santos et al., 2005, 2006; Luchese et al., 2007b) and by other compounds that oxidize sulfhydryl groups (Emanuelli et al., 1996; Barbosa et al., 1998). Furthermore, δ-ALA-D activity is extremely sensitive to situations associated with oxidative stress (Folmer et al., 2002; Nogueira et al., 2004; Luchese et al., 2007b). The relationship between δ-ALA-D and oxidative stress could be attributed, in this study, to the fact that cigarette smoke exposure reduced δ-ALA-D activity and the antioxidant agent $(PhSe)_2$ protected the enzyme against this inhibition, which indicates that cigarette smoke damage to δ-ALA-D is produced by an oxidative process. An alternative interpretation for the inhibition of δ-ALA-D activity is the direct action of metals, which are constituents of cigarette smoke, on the enzyme, inhibiting its activity.

Importantly, $(PhSe)_2$ protected against oxidative damage caused by cigarette smoke (P2) in the brains of rat pups, reinforcing the antioxidant potential of this compound. In fact, $(PhSe)_2$ ameliorated TBARS, NPSH levels, ascorbic acid content, and catalase and δ-ALA-D activities, demonstrating the role of this compound in protecting against oxidative stress. Accordingly, our research group has reported that $(PhSe)_2$ is a potent antioxidant agent (Nogueira et al., 2004) and presents higher thiol peroxidase activity than other diselenides in different tissues (Wilson et al., 1989; Meotti et al., 2004).

The lack of change in body weight gain in this experimental procedure reinforces the aspect of use of this compound as a possible alternative in the prevention of oxidative damage induced by cigarette smoke exposure in cerebral tissue. In addition, differences in the oxidative/antioxidative status in the brains of pup rats could be due to the experimental protocols of exposure, whereby in P2 the animals received a major cigarette smoking insult.

Therefore, the results of the current study indicate that $(PhSe)_2$ is a potent antioxidant against toxicity induced by passive smoking in the brain. Further studies are warranted to elucidate the precise mechanism of the $(PhSe)_2$ effect.

Acknowledgment

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Aebi, H. 1984. Catalase in vitro. *Meth. Enzymol.* 105:121–126.

Anand, C. V., Anand, U., and Agarwal, R. 1996. Anti-oxidant enzymes, gammaglutamyl transpeptidase and lipid peroxidation in kidney of rats exposed to cigarette smoke. *Indian J. Exp. Biol.* 34:486–488.

Anbarasi, K., Sabitha, K. E., and Devi, C. S. S. 2005. Lactate dehydrogenase isoenzyme patterns upon chronic exposure to cigarette smoke: Protective effect of Bacoside A. *Environ. Toxicol. Pharmacol.* 20:345–350.

Arteel, G. E., and Sies, H. 2001. The biochemistry of selenium and the glutathione system. *Environ. Toxicol. Pharmacol.* 10:153–158.

Avti, P. K., Kumar, S., Pathak, C. M., Vaiphei, K., and Khanduja, K. L. 2006. Smokeless tobacco impairs the antioxidant defense in liver, lung, and kidney of rats. *Toxicol. Sci.* 89:547–553.

Barbosa, N. B. V., Rocha, J. B. T., Zeni, G., Emanuelli, T., Beque, M. C., and Braga, A. L. 1998. Effect of organic selenium on δ-aminolevulinate

dehydratase from liver, kidney, and brain of adult rats. *Toxicol. Appl. Pharmacol.* 149:243–253.

Baskaran, S., Lakshimi, S., and Prasad, P. R. 1999. Effect of cigarette smoke on lipid peroxidation and antioxidant enzymes in albino rat. *Indian J. Exp. Biol.* 37:1196–1200.

Bowling, A. C., Schulz, J. B., Brow, R. H.,Jr., and Beal, M. F. 1993. Superoxide dismutase activity, oxidative damage, and mitochondrial energy metabolism in familial and sporadic amyotrophic lateral sclerosis. *J. Neurochem.* 61:2322–2325.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. *Anal. Biochem.* 72:248–254.

Brook, M., and Grimshaw, J. J. 1968. Vitamin C concentrations of plasma and leukocytes as related to smoking habit, age, and sex of human. *Am. J. Clin. Nutr.* 21:1254–1258.

Church, D. F., and Pryor, W. A. 1985. Free radicals chemistry of cigarette smoke and its toxicological implications. *Environ. Health Perspect.* 64:111–126.

Coyle, J. T., and Puttfarcken, P. 1993. Oxidative stress, glutamate, and neurodegenerative disorders. *Science* 262:689–695.

Dilsiz, N., Olcucu, A., Cay, M., Naziroglu, M., Cobanoglu, and D. 1999. Protective effects of selenium, vitamin C and vitamin E against oxidative stress of cigarette smoke in rats. *Cell Biochem. Funct.* 17:1–7.

Ellman, G. L. 1959. Tissue sulfhydryl groups. *Arch. Biochem.* 82:70–77.

Emanuelli, T., Rocha, J. B. T., Pereira, M. E., Porciuncula, L. O., Morsch, V. M., Martins, A. F., and Souza, D. O. 1996. Effect of mercuric and dimercaprol treatment on aminolevulinate dehydratase from brain, liver and kidney of adult mice. *Pharmacol. Toxicol.* 79:138–143.

Folmer, V., Soares, J. C. M., andRocha, J. B. T. 2002. Oxidative stress in mice is dependent on the free glucose content of the diet. *Int. J. Biochem. Cell Biol.* 34:1279–1285.

Genbacev-Krtolica, O. 2005. Highlight for phenols, quinolines, indoles, benzene and 2 cyclopenten-1-ones are oviduct toxicants in cigarette smoke, by Prue Talbot, Karen Riveles, and Ryan Rosa: List of tobacco-smoke constituents that are harmful for reproduction grows-passive smokers may be at risk. *Toxicol. Sci.* 86:4–5.

Gibson, K. D., Neuberger, A., and Scott, J. J. 1955. The purification and properties of delta-aminolevulinic acid dehydratase. *Biochem. J.* 61:618–628.

Halliwell, B., and Gutteridge, J. M. C. 2000. *Free radicals in biology and medicine*, 3rd ed. Oxford: Oxford University Press.

Helen, A., Rajasree, C. R., Krishnakumar, K., Augusti, K. T., and Vijayammal, P. L. 1999. Antioxidant role of oils isolated from garlic (Allium sativum Linn) and onion (Allium cepa Linn) on nicotine-induced lipid peroxidation. *Vet. Hum. Toxicol.* 41:316–319.

Hilbert, J., and Mohsenin, V. 1996. Adaptation of lung antioxidants to cigarette smoking in humans. *Chest* 110:916–920.

Horning, D. H., and Glatthaar, B. E. 1985. Vitamin C and smoking: Increased requirements of smokers. *Int. J. Vit. Nutr. Res.* 27:139–155.

Jacques-Silva, M. C., Nogueira, C. W., Broch, L. C., Flores, E. M. M., and Rocha, J. B. T. 2001. Diphenyl diselenide and ascorbic acid changes deposition of selenium and ascorbic acid in liver and brain of mice. *Pharmacol. Toxicol.* 88:119–125.

Kallner, A. B., Hartmann, D., and Hornig, D. H. 1981. On the requirements of ascorbic acid in man: Steady state turnover and body pool in smokers. *Am. J. Clin. Nutr.* 34:1347–1355.

Luchese, C., Stangherlin, E. C., Ardais, A. P., Nogueira, C. W., and Santos, F. W. 2007a. Diphenyl diselenide prevents oxidative damage induced by cigarette smoke exposure in lung of rat pups. *Toxicology* 230:189–196.

Luchese, C., Zeni, G., Rocha, J. B. T., Nogueira, C. W., and Santos, F. W. 2007b. Cadmium inhibits δ-aminolevulinate dehydratase from rat lung in vitro: Interaction with chelating and antioxidant agents. *Chem. Biol. Interact.* 165:127–137.

Meotti, F. C., Borges, V. C., Zeni, G., Rocha, J. B. T., and Nogueira, C. W. 2003. Potential renal and hepatic toxicity of diphenyl diselenide, diphenyl ditelluride and ebselen for rats and mice. *Toxicol. Lett.* 143:9–16.

Meotti, F. C., Stangherlin, E. C., Zeni, G., Nogueira, C. W., and Rocha, J. B. T. 2004. Protective role of aryl and alkyl diselenides on lipid peroxidation. *Environ. Res.* 94:276–282.

Misra, H. P., and Fridovich, I. 1972. The role of superoxide anion in the autoxidation of epinephrine and simple assay for superoxide dismutase. *J. Biol. Chem.* 247:3170–3175.

Nogueira, C. W., Meotti, F. C., Curte, E., Pilissao, C., Zeni, G., and Rocha, J. B. T. 2003. Investigations into the potential neurotoxicity induced by diselenides in mice and rats. *Toxicology* 183:29–37.

Nogueira, C. W., Zeni, G., and Rocha, J. B. T. 2004. Organoselenium and organotellurium compounds: Toxicology and pharmacology. *Chem. Rev.* 104:6255–6285.

Ohkawa, H., Ohishi, N., and Yagi, K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* 95:351–358.

Olanow, C. W. 1990. Oxidation reactions in Parkinson's disease. *Neurology* 40:S32–S37.

O'Neill, C. A., Halliwell, B., Vander Vliet, A., Davis, P. A., Packer, L., Tritschler, H., Strohman, W. J., Rieland, T., Cross, C. E., and Reznick, A. Z. 1994. Aldehyde-induced protein modifications in human plasma: protection by glutathione and dihydrolipoic acid. *J. Lab. Clin. Med.* 124:359–370.

Park, E. M., Park, Y. M., and Gwak, Y. S. 1998. Oxidative damage in tissues of rats exposed to cigarette smoke. *Free. Radic. Biol. Med.* 25:79–86.

Paulmier, C. 1986. *Selenium reagents and intermediates in organic synthesis.* Oxford: Pergamon Press.

Polidori, M. C., Mecocci, P., Stahl, W., and Sies, H. 2003. Cigarette smoking cessation increases plasma levels of several antioxidant micronutrients and improves resistance towards oxidative challenge. *Br. J. Nutr.* 90:147–150.

Pryor, W. A., and Stone, K. 1993. Oxidants in cigarette smoke. *Ann. N. Y. Acad. Sci.* 686:12–28.

Reddy, S., Finkelstein, E. I., Wong, P., Phung, A., Cross, C. E., and Van der Vliet, A. 2002. Identification of glutathione modifications by cigarette smoke. *Free Radic. Biol. Med.* 33:1490–1498.

Richards, G. A., van Antwerpen, V. L., Hunter, S., and Theron, A. J. 1996 . Ageing and cigarette smoking are associated with decreased glutathione levels in humans. *S. Afr. J. Sci.* 92:445–447.

Rocha, J. B. T., Pereira, M. E., Emanuelli, T., Christofari, R. S., and Souza, D. O. 1995. Effect of treatment with mercury chloride and lead acetate during the second stage of rapid postnatal brain growth on delta-aminolevulinic acid dehydratase (ALA-D) activity in brain, liver, kidney and blood of suckling rats. *Toxicology* 100:27–37.

Rodrigues, A. L. S., Rocha, J. B. T., Pereira, M. E., and Souza, D. O. 1996. Delta-aminolevulinic acid dehydratase activity in weanling and adult rats exposed to lead acetate. *Environ. Contam. Toxicol.* 57:47–53.

Rossato, J. I., Ketzer, L. A., Centurião, F. B., Silva, S. J. N. Ludtke, D. S., Zeni, G., Braga, A. L., Rubin, M. A., and Rocha, J. B. T. 2002. Antioxidant propeties of new chalcogenides against lipid peroxidation in rat brain. *Neurochem. Res.* 27:297–303.

Sassa, S. 1982. Delta-aminolevulinic acid dehydratase assay. *Enzyme* 28:133–145.

Santos, F. W., Zeni, G., Rocha, J. B. T., Weis, S. N., Fachinetto, J. M., Favero, A. M., and Nogueira, C. W. 2005. Diphenyl diselenide reverses cadmium-induced oxidative damage on mice tissues. *Chem. Biol. Interact.* 151:159–165.

Santos, F. W., Rocha, J. B. T., and Nogueira, C. W. 2006. 2,3-Dimercaptopropanol, 2,3-dimercaptopropane-1-sulfonic acid and meso-2,3-dimercaptosiccinic acid increase lead-induced inhibition of δ-aminolevulinate dehydratase in vitro and ex vivo. *Toxicol. In Vitro* 20:317–323.

Sobczak, A., Golka, D., and Szoltysek-Boldys, I. 2004. The effects of tobacco smoke on plasma alpha- and gamma-tocopherol levels in passive and active cigarette smokers. *Toxicol. Lett.* 151:429–437.

US Department of Health and Human Services (US DHHS). 1989. *Reducing the health consequences of smoking: 25 years of progress. A report of the surgeon general*, DHHS Publication No. (CDC)89–8411. Washington, DC: US DHSS.

Wilson, S. R., Zucker, P. A., Huang, R. C., and Spector, A. 1989. Development of synthetic compounds with glutathione peroxidase activity. *J. Am. Chem. Soc.* 111:5936.

World Health Organization (WHO). 1992. *Environmental health criteria 134: Cadmium. International program on chemical safety (IPCS) monograph.* Geneva: WHO.

Yu, B. P. 1994. Cellular defenses against damage from reactive oxygen species. *Physiol. Rev.* 74:139–162.

4.4. Manuscrito 1:

Disseleneto de Difenila tem Atividades Dehidroascorbato Redutase e Glutationa S-transferase-*like*

DIPHENYL DISELENIDE HAS DEHYDROASCORBATE REDUCTASE AND GLUTATHIONE S-TRANSFERASE-LIKE ACTIVITIES

Cristiane Luchese, Cristina W. Nogueira

Submetido à *Archives of Biochemistry and Biophysics*

Diphenyl Diselenide has Dehydroascorbate Reductase and Glutathione S-Transferase-Like Activities

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Running read: $(PhSe)_2$ has DHA Reductase and GST-Like Activities

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Abstract

The antioxidant action of diphenyl diselenide [$(PhSe)_2$] is attributed to the mechanism by which $(PhSe)_2$ presents pharmacological activity. However, the exact mechanism involved in the antioxidant effect of $(PhSe)_2$ has not been completely elucidated, but it has been reported to show glutathione peroxidase mimetic activity. In the present study, the mechanisms involved in the antioxidant effect of $(PhSe)_2$ (1 – 50 µM) were studied. Besides, dehydroascorbate (DHA) reductase- and glutathione S-transferase (GST)-like activities, 2,2'-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical-scavenging activities and the protection against the oxidation of Fe^{2+} were evaluated. $(PhSe)_2$ at concentrations equal or greater than 5 µM presented DHA reductase- and GST-like activities. $(PhSe)_2$ was not scavenger of DPPH and ABTS radical and did not protect against the oxidation of Fe^{2+}. In conclusion, these results clearly indicated that DHA reductase- and GST-like activities are the mechanisms involved in the antioxidant effect of $(PhSe)_2$.

Keywords: Diphenyl Diselenide, Dehydroascorbate Reductase-like, Glutathione S-Transferase-like, Selenium, Antioxidant, Scavenger.

Introduction

The concept that selenium-containing molecules may be better nucleophiles (and therefore antioxidants) than classical antioxidants have led to design synthetic organoselenium compounds [1]. In vitro studies have suggested that organoselenium compounds can be considered potential antioxidant compounds [2,3].

Diphenyl diselenide [$(PhSe)_2$], an organoselenium compound, has been shown to reduce lipid peroxidation induced by a variety of oxidants [4,5]. In addition, $(PhSe)_2$ has many pharmacological properties such as antiulcer [6], antiinflammatory and antinociceptive [7], as well as anti-hyperglycemic [8]. Moreover, the antioxidant property of $(PhSe)_2$ has been shown in several animal models of oxidative stress [3,10-17]. The antioxidant action of $(PhSe)_2$ is attributed to the mechanism by which $(PhSe)_2$ presents pharmacological activity.

However, the exact mechanism involved in the antioxidant effect of $(PhSe)_2$ has not been completely elucidated. Of particular importance, the antioxidant activity of various organoselenium compounds, such as ebselen and $(PhSe)_2$ seems to be related, at least in part, to their glutathione peroxidase (GPx) mimetic effect [5,18]. Evidences in the literature indicate that $(PhSe)_2$ displays thiol peroxidase activity [3,19] and acts in different antioxidant lines of defense [8, 12-17], clearly showed by the efficiency of this organoselenium compound.

In addition, Jung et al. [20] has demonstrated that besides the GPx-like activity of ebselen, the thioltransferase and dehydroascorbate reductase-like activities seem to be related to the antioxidant effect of this compound.

The purpose of the present study was to investigate the mechanisms involved in the antioxidant effect of $(PhSe)_2$ to elucidate the well documented efficacy of this compound as a potent agent with several pharmacological properties.

Materials and methods

Chemicals

Reduced glutathione (GSH), 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma (St. Louis, MO, USA). 1-Chloro-2,4-dinitrobenzene (CDNB) was purchased from Aldrich Chemical Co (USA). Dehydroascorbate (DHA) was prepared by the oxidation of L-ascorbic acid with bromine as described previously (Wells et al., 1995). $(PhSe)_2$ was prepared in our laboratory according to the method described in the literature [21].

Analyses of the ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR spectrum showed that $(PhSe)_2$ obtained presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of the compound (99.9%) was determined by GC/HPLC. This drug was dissolved in dimetilsulfoxide (DMSO), which was obtained from a standard commercial supplier. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Dehydroascorbate (DHA) reductase-like assay

DHA reductase activity of $(PhSe)_2$ was assayed as described previously [22,23] with minor modifications. In brief, $(PhSe)_2$ (1–50 μM) was incubated (1–2 min) in 100 mM sodium phosphate buffer, pH 6.9, at 25 ºC in the presence of GSH (1–3 mM). DHA reductase activity was initiated by adding DHA (0.5 mM) to a final volume of 1.0 ml. L-ascorbic acid regeneration was recorded at 265 nm. A blank without $(PhSe)_2$ was run, and the difference gave the $(PhSe)_2$ DHA reductase activity in nmol/min using the molar extinction coefficient of ascorbic acid of 14,700 $cm^{-1}M^{-1}$. Ebselen (1–50 μM) was used as a positive control.

At the end of DHA reductase-like assay, $(PhSe)_2$ was determined in the samples. Samples (1.0 ml - final volume of DHA reductase-like assay) were mixed to ethyl acetate in

the ratio of 2:1 (v/v) to tube. The extraction was performed by vortex-mixing the tubes for 3 min. After, samples were centrifuged at 3000 for 5 min. Supernatants were transferred to a clean test tube and 1 µl aliquot was injected into chromatographic system for analysis. Qualitative analysis of $(PhSe)_2$ in samples were conducted by gas chromatography (CG 2010 Shimadzu®) associated to flame ionized detector system (FID) and using a 5% diphenyl/95% dimethyl column, 30m×0.25mm×0.25µm, from Restek®. The limit of detection (LOD) for $(PhSe)_2$ was 0.5 µg/ml. $(PhSe)_2$ standard was utilized with the finality to obtain the retention time.

Glutathione S-Transferase (GST)-like assay

The reaction of GSH with CDNB is typically the preferred system used to measure the catalysis imparted by naturally occurring glutathione S-transferases [24]. Reaction of $(PhSe)_2$ with CDNB demonstrates GST-like activity. $(PhSe)_2$ (1–50 µM) was incubated with 2.0 mM GSH at 25°C for 3 min. The reaction was initiated by adding 1.0 mM CDNB to a final volume of 1.0 ml in 100 mM sodium phosphate buffer, pH 6.9 and recorded for 3 min at 340 nm. A blank without $(PhSe)_2$ was included and the difference was expressed as ΔA/min. Ebselen (1–50 µM) was used as a positive control.

DPPH radicals

The DPPH stable radical was performed in accordance with Choi et al. [25]. Briefly, 50 µM DPPH was added to a medium containing $(PhSe)_2$ (10–50 µM). The media were incubated for 30 min at 25ºC in dark. The decrease in absorbance was measured at 518 nm, depicting the scavenging activity of $(PhSe)_2$ against DPPH radical. Ascorbic acid (10–50 µM) was used as a positive control to determine the maximal decrease in DPPH absorbance. Results are expressed as percentage of the blank (without compound).

ABTS radicals

The determination of the ABTS radical scavenging effect of $(PhSe)_2$ was performed according to the method of Re et al. [26], with some modifications. Initially, the ABTS radical was generated by reacting 7 mM ABTS solution in water with 140 mM potassium persulfate in the dark for 12-16 h. In the day of the assay, the pre-formed ABTS radical solution was diluted 1:88 (1 ml ABTS radical + 87 ml 10 mM potassium phosphate buffer, pH 7.0). Briefly, ABTS radical was added to a medium containing $(PhSe)_2$ (10–50 µM). The media were incubated for 30 min at 25ºC. The decrease in absorbance was measured at 734 nm, depicting the scavenging activity of $(PhSe)_2$ against ABTS radical. Ascorbic acid (10–50 µM) was used as a positive control to determine the maximal decrease in ABTS absorbance. Results are expressed as percentage of the blank (without compound).

Autooxidation of Fe^{2+}

The interaction of $(PhSe)_2$ with iron was evaluated as described by Yoshino and Murakami [27], with some modifications. The reaction was started by the addition of $FeSO_4$ in samples of 2 ml containing 10 mM Tris-HCl pH 7.1, 0.1 mM $FeSO_4$ and $(PhSe)_2$ (10–50 µM) in the absence and the presence of 0.1 mM isocitrate. Aliquots of 0.6 ml were mixed with 0.3 ml of 1 mM 1,10-phenanthroline at 40 min and the absorbance at 540 nm was measured. Ascorbate (0.1 mM) was used as a positive control. Values are expressed in percentage of isocitrate in relation to the control values without $(PhSe)_2$.

Statistical analysis

Data are expressed as means ± S.D. The statistical analysis was performed using a one-way ANOVA followed by Duncan's test. Values of $p < 0.05$ were considered statistically significant.

Results

DHA reductase-like activity

Ebselen, a positive control, at concentrations equal or greater than 5 μM presented DHA reductase-like activity dependent on GSH (data not shown). $(PhSe)_2$ acted as a GSH-dependent DHA reductase, and the rate of reduction was closely proportional to the concentration of GSH and $(PhSe)_2$. At concentrations equal or greater than 5 μM, $(PhSe)_2$ reduced DHA to L-ascorbic acid (Figure 1). The DHA reductase-like activity of $(PhSe)_2$ was similar to that of ebselen. At the end of the DHA reductase-like assay, $(PhSe)_2$ was detected by GC.

GST-like activity

Ebselen, a positive control, at concentrations equal or greater than 10 μM presented GST-like activity in the presence of GSH (data not shown). $(PhSe)_2$, at concentrations equal or greater than 5 μM, demonstrated GST-like activity in the presence of GSH (Figure 2). The reaction rate is essentially proportional to $(PhSe)_2$ concentration. The GST-like activity of $(PhSe)_2$ was superior to that of ebselen.

DPPH radical scavenging activity

Ascorbic acid, at the concentration equal or greater than 10 μM, showed DPPH radical-scavenging activity. $(PhSe)_2$ at all concentrations tested did not present DPPH radical-scavenging activity (Table 1).

ABTS radical

Ascorbic acid at the concentration of 10 μM showed ABTS radical-scavenging activity. $(PhSe)_2$ at all concentrations did not show ABTS radical-scavenging activity (Table 1).

Autooxidation of Fe^{2+}

$(PhSe)_2$ (10 – 50 μM) alone did not affect the reduced state of iron (data not shown). Isocitrate stimulated the autooxidation of Fe^{2+} to Fe^{3+} ion and ascorbate was able to maintain iron in the reduced form, inhibiting the isocitrate-mediated oxidation of Fe^{2+} ion effectively. $(PhSe)_2$ (10 – 50 μM) was not effective in protecting against the oxidation of Fe^{2+} (Figure 3).

Discussion

In this study we report, for the first time, that $(PhSe)_2$ in the presence of glutathione, has DHA reductase- and GST-like activities and, thereby, may significantly contribute to the well documented efficacy of the compound as a potent antioxidant agent. However, we could demonstrate that the compound did not present DPPH and ABTS radical scavenging activities and did not protect against autooxidation of Fe^{2+}.

DHA reductase is an enzyme that catalyzes the reduction of DHA to ascorbic acid [22,28]. Our findings presented that $(PhSe)_2$ at low concentration (5 μM) presented DHA reductase-like activity. Accordingly, in vivo studies have revealed that rodents treated with $(PhSe)_2$ showed an increase in the levels of ascorbic acid [12-17]. Therefore, the increase of ascorbic acid content in $(PhSe)_2$ treatment, found in previous studies, is likely to occur due to the DHA reductase-like activity of the compound, increasing the reduction of DHA to ascorbic acid. Data in the literature have demonstrated that other organoselenium compounds, such as ebselen (10 μM) presented DHA reductase-like activity [20]. Indeed, our data revealed that the DHA reductase-like activity of $(PhSe)_2$ was similar to that of ebselen.

Based on the mechanism proposed by Jung et al. [20] for the DHA reductase-like activity of ebselen, we suggested a similar mechanism for $(PhSe)_2$ DHA reductase-like activity (Scheme 1). $(PhSe)_2$ is reduced by GSH to its selenol form (phenylselenol) giving the oxidized form glutathione disulfide, GSSG (Scheme 1, reaction **1**). Phenylselenol reacts with DHA to form phenylselenohemiketal (Scheme 1, reaction **2**), which reacts with another molecule of GSH to release L-ascorbic acid and the intermediate, phenylseleno-glutathione sulfide (Scheme 1, reaction **3**). This compound then reacts with another GSH to regenerate $(PhSe)_2$ and GSSG (Scheme 1, reaction **4**). Intracellular systems rely on the GSH regeneration by NADPH and glutathione reductase (GR) activity. Based on the reaction stoichiometry and on the detection of $(PhSe)_2$ after DHA reductase-like assay, we suggested that this is a catalytic cycle.

GST, also known as phase II enzymes, is widely distributed catalyzing and binding proteins which promote the conjugation of GSH with a variety of reactive electrophilic compounds resulting in the formation of substances easily excreted from the body [29,30]. Moreover, several authors have reported that GST is an antioxidant defense and serves to protect tissues against oxidative stress [31-33]. The current study demonstrated that $(PhSe)_2$ presented GST-like activity. Thus, as GST is reported as an antioxidant enzyme, we assumed that the GST-like activity of $(PhSe)_2$ could be involved in the antioxidant effect of this compound. According to Jung et al. [20], ebselen (10 µM), an organoselenuim compound, presented GST-like activity. In fact, our data revealed that the GST-like activity of $(PhSe)_2$ was superior to that of ebselen.

The findings of the present study clearly indicated that the antioxidant effect of $(PhSe)_2$ is related neither to DPPH nor to ABTS radical scavenging activities. Stable radicals DPPH and ABTS have been widely used for the determination of primary antioxidant activities of pure antioxidant compounds, plant and fruit extracts, and food materials [34,35].

We can assure that DPPH and ABTS radical scavenging activities are not the mechanisms by which $(PhSe)_2$ displayed antioxidant activity.

In addition, it is known that the formation of ROS is closely related to the redox state of transition metals, such as iron [36]. Superoxide anion is readily produced through the one-electron reduction of oxygen by ferrous ion, and is largely dismuted into hydrogen peroxide by enzymatic and nonenzymatic mechanisms [37]. Hydrogen peroxide is further converted to hydroxyl radical by the Fenton reaction, which requires reduced iron [36]. Reduced iron also binds to molecular oxygen, and forms perferryl ion (Fe^{2+}-O_2) [38]. Hydroxyl radical and perferryl ion are highly reactive and act as the actual initiating species for cellular lipid peroxidation [37]. The effective inhibition of the isocitrate-mediated enhancement of Fe^{2+} autooxidation results in decreased formation of perferryl ion by keeping the iron at a reduced state. $(PhSe)_2$ was not effective in protecting against the oxidation of Fe^{2+}. These results discarded the protection against oxidation of Fe^{2+} as a mechanism involved in $(PhSe)_2$ antioxidant action.

In conclusion, our results strongly indicated that the antioxidant effect of $(PhSe)_2$ is probably due to DHA reductase- and GST-like activities. However, this effect was not related to DPPH and ABTS radical scavenging activities as well as to the protection against the oxidation of Fe^{2+}.

Acknowledgements

The financial support by FAPERGS, CAPES (Edital Pró-Equipamentos/2007) and CNPq is gratefully acknowledged. C.W.N. is recipient of CNPq fellowship.

References

[1] G.E. Arteel, H. Sies, The biochemistry of selenium and the glutathione system, Environ. Toxicol. Pharmacol. 10 (2001) 153–158.

[2] M.J. Parnham, E. Graf, Pharmacology of synthetic organic selenium compounds, Prog. Drug Res. 36 (1991) 9–47.

[3] F.C. Meotti, E.C. Stangherlin, G. Zeni, J.B.T. Rocha, C.W. Nogueira, Protective role of aryl and alkyl diselenides on lipid peroxidation, Environ. Res. 94 (2004) 276–282.

[4] J.I. Rossato, L.A. Ketzer, F.B. Centuriao, S.J. Silva, D.S. Ludtke, G. Zeni, A.L. Braga, M.A. Rubin, J.B.T. Rocha, Antioxidant properties of new chalcogenides against lipid peroxidation in rat brain, Neurochem. Res. 27 (2002) 297–303.

[5] C.W. Nogueira, G. Zeni, J.B.T. Rocha, Organoselenium and organotellurium compounds: toxicology and pharmacology, Chem. Rev. 104 (2004) 6255 – 6286.

[6] L. Savegnago, M. Trevisan, D. Alves, J.B.T. Rocha, C.W. Nogueira, G. Zeni, Antisecretory and antiulcer effects of diphenyl diselenide, Environ. Toxicol. Pharmacol. 21 (2006) 86 – 92.

[7] L. Savegnago, L.G. Pinto, C.R. Jesse, D. Alves, J.B.T. Rocha, C.W. Nogueira, G. Zeni, Antinociceptive properties of diphenyl diselenide: Evidences for the mechanism of action, Eur. J. Pharmacol. 555 (2007) 129 – 138.

[8] N.B.V. Barbosa, J.B.T. Rocha, D.C. Wondracek, J. Perottoni, G. Zeni, C.W. Nogueira, Diphenyl diselenide reduces temporarily hyperglycemia: Possible relationship with oxidative stress, Chem.-Biol. Interact. 163 (2006) 230 – 238.

[9] F.W. Santos, T. Oro, G. Zeni, J.B.T. Rocha, P.C. do Nascimento, C.W. Nogueira, Cadmium induced testicular damage and its response to administration of succimer and diphenyl diselenide in mice, Toxicol. Lett. 152 (2004) 255 – 263.

[10] F.W. Santos, G. Zeni, J.B.T. Rocha, P.C. do Nascimento, M.S. Marques, C.W. Nogueira, Efficacy of 2,3-dimercapto-1-propanesulfonic acid (DMPS) and diphenyl diselenide on cadmium induced testicular damage in mice, Food Chem. Toxicol. 43 (2005) 1723 – 1730.

[11] F.W. Santos, G. Zeni, J.B.T. Rocha, S.N. Weis, J.M. Fachinetto, A.M. Favero, C.W. Nogueira, Diphenyl diselenide reverses cadmium-induced oxidative damage on mice tissues Chem.-Biol. Interact. 151 (2005) 159 – 165.

[12] L.P. Borges, R. Brandao, B. Godoi, C.W. Nogueira, G. Zeni, Oral administration of diphenyl diselenide protects against cadmium-induced liver damage in rats, Chem.-Biol. Interact. 171 (2008) 15 – 25.

[13] C. Luchese, R. Brandao, R. de Oliveira, C.W. Nogueira, F.W. Santos, Efficacy of diphenyl diselenide against cerebral and pulmonary damage induced by cadmium in mice, Toxicol. Lett. 173 (2007) 181 – 190.

[14] C. Luchese, E.C. Stangherlin, A.P. Ardais, C.W. Nogueira, F.W. Santos, Diphenyl diselenide prevents oxidative damage induced by cigarette smoke exposure in lung of rat pups, Toxicology 230 (2007) 189 – 196.

[15] C. Luchese, P. Pinton, C.W. Nogueira, Brain and lungs of rats are differently affected by cigarette smoke exposure: antioxidant effect of an organoselenium compound, Pharmacol. Res. 59 (2009) 194 – 201

[16] C. Luchese, E.C. Stangherlin, B.M. Gay, C.W. Nogueira, Antioxidant effect of diphenyl diselenide on oxidative damage induced by smoke in rats: involvement of glutathione, Ecotox. Environ. Safe. 72 (2009) 248 – 254.

[17] M. Prigol, C. Luchese, C.W. Nogueira, Antioxidant effect of diphenyl diselenide on oxidative stress caused by acute physical exercise in skeletal muscle and lungs of mice, Cell Biochem. Funct. (2009) DOI:10.1002/cbf.1559.

[18] R. Zhao, A. Holmgren, Ebselen is a dehydroascorbate reductase mimic, facilitating the recycling of ascorbate via mammalian thioredoxin systems, Antioxid. Redox. Signal. 6 (2004) 99 – 104.

[19] S.R. Wilson, P.A. Zucker, R.C. Huang, A. Spector, Development of synthetic compounds with glutathione peroxidase activity, J. Am. Chem. Soc. 111 (1989) 5936.

[20] C.H. Jung, M.P. Washburn, W.W. Wells. Ebselen has dehydroascorbate reductase and thioltransferase-like activities, Biochem. Biophys. Res. Com. 291 (2002) 550 – 553.

[21] C. Paulmier, Selenium reagents and intermediates, in: Organic Synthesis, Oxford: Pergamon, 1986.

[22] W.W. Wells, D.P. Xu, M.P. Washburn, Glutathione – dehydroascorbate oxidoreductases, Methods Enzymol. 252 (1995) 30 – 38.

[23] M.P. Washburn, W.W. Wells, Identification of the dehydroascorbic acid reductase and thioltransferase (glutaredoxin) activities of bovine erythrocytes glutathione peroxidase, Biochem. Biophys. Res. Commun. 257 (1999) 567 – 571

[24] W.H. Habig, M.J. Pabst, W.B. Jakoby, Glutathione S-Transferases. The first enzymatic step in mercapturic acid formation, J. Biol. Chem. 249 (1974) 7130 – 7139.

[25] C.W. Choi, S.C. Kim, S.S. Hwang, B.K. Choi, H.J. Ahn, M.Y. Lee, S.H. Park, S.K. Kim, Antioxidant activity and free radical scavenging capacity between Korean medicinal plants and flavonoids by assay- guided comparison, Plant Sci. 153 (2002) 1161 – 1168.

[26] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, Antioxidant activity applying an improved ABTS radical cation decolorization assay, Free Radic. Biol. Med. 26 (1999) 1231 – 1237.

[27] M. Yoshino, K. Murakami, Interaction of iron with polyphenolic compounds: application to antioxidant characterization, Anal. Biochem. 257 (1998) 40 – 44.

[28] D.P. Xu, M.P. Washburn, G.P. Sun, W.W. Wells, Purification and characterization of a glutathione dependent dehydroascorbate reductase from human erythrocytes, Biochem. Biophys. Res. Commun. 221 (1996) 117 – 121.

[29] L.F. Chasseaund, The role of glutathione and glutathione S-transferase in the chemicals carcinogens and other electrophilic agents, Adv. Cancer Res. 29 (1979) 175 – 274.

[30] I. Cervello, A. Lafuente, M. Giralt, J. Mallol, Enhanced glutathione S-transferase (GST) activity in pregnantrats treated with benzo(a)pyrene, Placenta 13 (1992) 273 – 280.

[31] E. Mosialou, R. Morgenstern, Activity of rat liver microsomal glutathione transferase toward products of lipid peroxidation and studies of the effect of inhibitors on glutathione-dependent protection against lipid peroxidation., Arch. Biochem. Biophys. 275 (1989) 289 – 294.

[32] J.D. Hayes, D.J. Pulford, The glutathione S-transferases upper gene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drugresistance, Crit. Rev. Biochem. Mol. Biol. 30 (1995) 445 – 600.

[33] H. Fiander, H. Schneider, Compounds that induce isoforms of glutathione S- transferase with properties of a critical enzyme in defense against oxidative stress, Biochem. Biophys. Res. Commun. 262 (1999) 591 – 595.

[34] C.W. Chen, C.T. Ho, Antioxidant properties of polyphenols extracted from green and black tea, J. Food Lipids 2 (1995) 35 – 46.

[35] R. Apak, K. Guclu, M. Ozyurek, S.E. Celik, Mechanism of antioxidant capacity assays and the CUPRAC (cupric ion reducing antioxidant capacity) assay, Microchim. Acta 160 (2008) 413 – 419.

[36] B. Halliwell, J.M.C. Gutteridge, Role of free-radicals and catalytic metal-ions in human-disease – an overview, Meth. Enzymol. 186 (1990) 1 – 85.

[37] I. Fridovich, Superoxide dismutases - an adaptation to a paramagnetic gas, J. Biol. Chem. 264 (1989) 7761 – 7764.

[38] D.M. Miller, S.D. Aust, Studies of ascorbate-dependent, iron-catalyzed lipid peroxidation, Arch. Biochem. Biophys. 271 (1989) 113 – 119.

Figure Legends

Figure 1 – DHA reductase-like activity of $(PhSe)_2$. $(PhSe)_2$ (1 - 50 μM) was incubated in 100 mM sodium phosphate buffer, pH 6.9, at 25 ºC in the presence of (■) 1 mM, (●) 2 mM and (▲) 3 mM GSH. DHA reduction was measured by absorbance increase at 265 nm. Values are the means ± S.D. of three or more determinations. (*) Denotes $p < 0.05$ as compared to the blank (without $(PhSe)_2$) (one-way ANOVA/Duncan).

Figure 2 –GST-like activity of $(PhSe)_2$. $(PhSe)_2$ (1 - 50 μM) was incubated at 25 ºC in the presence of 2 mM GSH and 1 mM CDNB in 100 mM sodium phosphate buffer, pH 6.9. The reaction was measured by absorbance increase at 340 nm. Values are the means ± S.D. of three or more determinations. (*) Denotes $P < 0.05$ as compared to the blank (B - without $(PhSe)_2$) (one-way ANOVA/Duncan).

Figure 3 – Effect of $(PhSe)_2$ (10 - 50 μM) on autooxidation of ferrous ion in the absence and presence of isocitrate. Ascorbate was used as positive control. Iron autooxidation was evaluated at 40 min. Results are expressed as percentage of control. Values are the means ± SD of three or more independent experiments. (*) Denotes $p < 0.05$ as compared to the control. (#) Denotes $p < 0.05$ as compared to isocitrate.

Scheme Legend

Scheme 1 - Mechanism of $(PhSe)_2$ DHA reductase-like activity.

Table

Table 1- Scavenging activity of $(PhSe)_2$ and ascorbic acid on DPPH and ABTS radical.

	DPPH Radical		**ABTS Radical**	
µM	$(PhSe)_2$	Ascorbic Acid	$(PhSe)_2$	Ascorbic Acid
0	100.0 ± 5.0	100.0 ± 5.0	100.0 ± 5.0	100.0 ± 5.0
10	103.7 ± 5.8	85.6 ± 6.5*	95.0 ± 7.0	64.0 ± 2.8*
20	105.3 ± 7.4	68.8 ± 2.5*	94.0 ± 6.1	32.0 ± 11.3*
30	106.6 ± 8.0	67.0 ± 1.0*	98.0 ± 3.6	4.9 ± 0.2*
40	104.0 ± 5.6	63.3 ± 5.1*	97.3 ± 6.0	2.2 ± 0.2*
50	100.0 ± 2.0	50.3 ± 7.8*	100.7 ± 6.4	2.4 ± 0.3*

Data are reported as mean ± S.D. of four independent experiments. 2,2'-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) are expressed as percentage of blank (without compound – 0). (*) Denotes $p < 0.05$; as compared to the blank (0) (one-way ANOVA/Duncan).

Figures

Figure 1

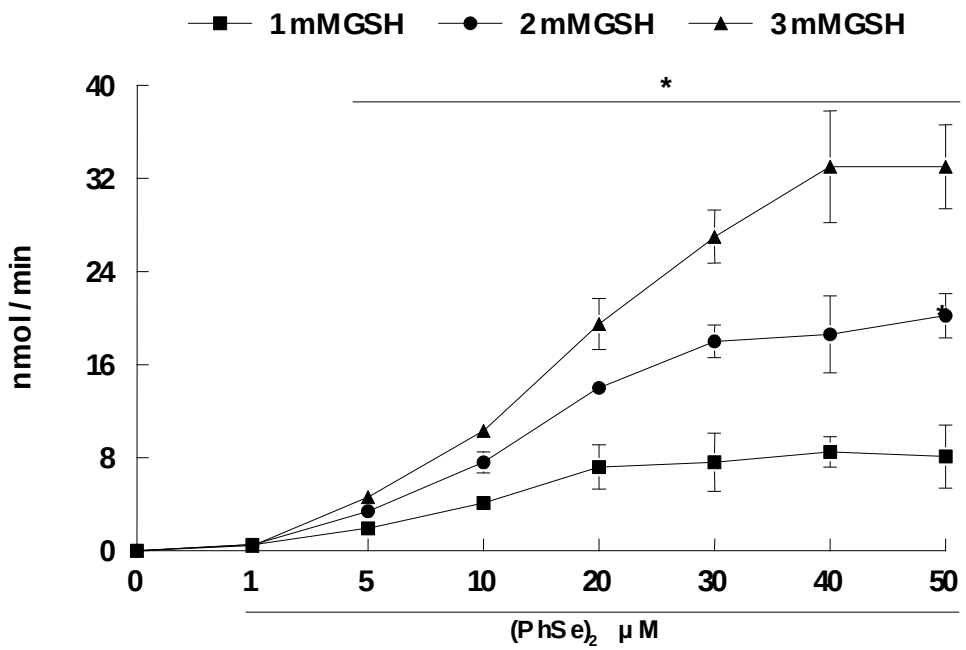


Figure 2

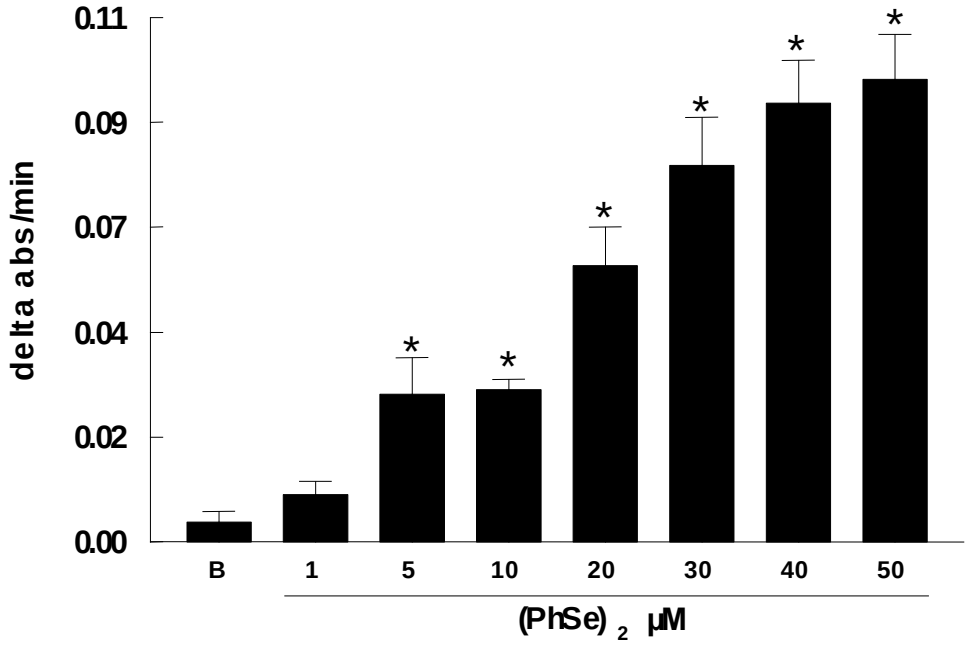
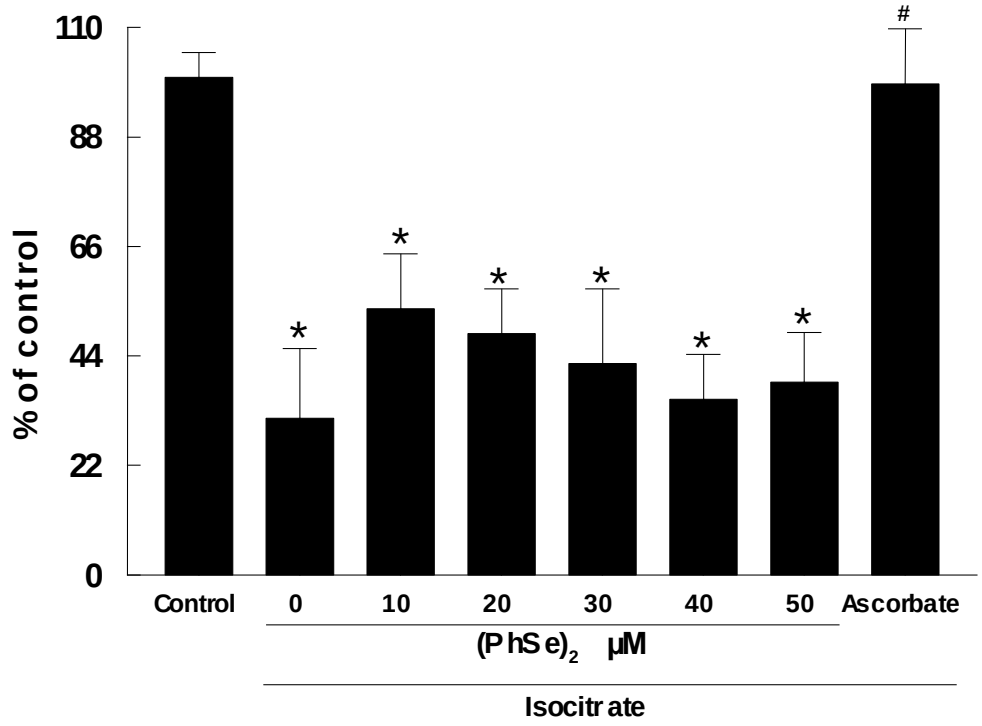


Figure 3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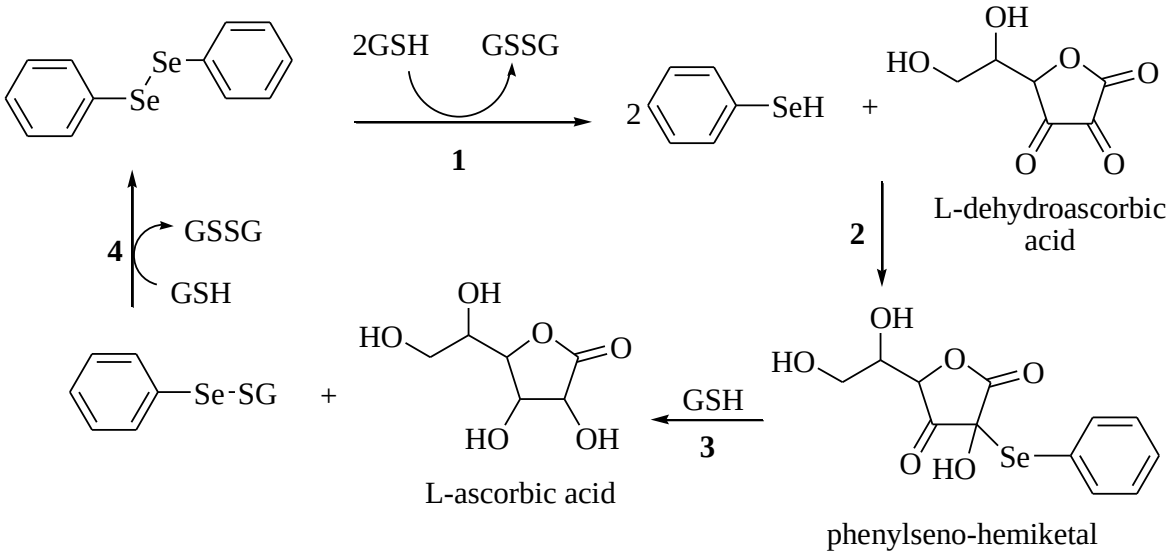
Scheme

Scheme 1



5. DISCUSSÃO

A exposição passiva à fumaça do cigarro é um fator de risco para o desenvolvimento de diversas doenças infantis, tais como doenças respiratórias e outras síndromes (Hofhuis et al., 2003). As crianças são mais suscetíveis aos danos causados pela exposição passiva à fumaça do cigarro do que os adultos, porque seus túbulos brônquicos são menores, seu sistema imune é menos desenvolvido e apresentam um metabolismo diferente quando comparadas com os adultos (Dejin-Karlsson et al., 1998; Who, 1999; Tutka et al., 2002). Durante a lactação, a exposição passiva à fumaça do cigarro pode aumentar o risco de doenças e síndromes infantis tais como a asma e bronquites (Klonoff-Cohen et al., 1995).

Dessa forma, no **Artigo 1**, verificou-se o efeito da exposição passiva à fumaça de um, dois e três cigarros na primeira, segunda e terceira semanas de vida, respectivamente, no pulmão de ratos jovens. O presente trabalho demonstrou claramente que a exposição passiva à fumaça do cigarro, neste protocolo experimental, induziu um estresse oxidativo nos pulmões dos ratos jovens não somente por aumentar a peroxidação lipídica, mas também por inibir a atividade da δ-aminolevulinato desidratase (δ-ALA-D) e por reduzir as defesas antioxidantes não-enzimáticas (níveis de SHNP e ácido ascórbico). Entretanto, a exposição passiva à fumaça do cigarro não alterou as defesas antioxidantes enzimáticas (atividade da SOD e CAT) nos pulmões dos ratos jovens.

Além dos pulmões, o cérebro também é extremamente vulnerável ao estresse oxidativo e aos danos causados pela exposição à fumaça do cigarro. Dessa forma, no **Artigo 3**, examinou-se o efeito da exposição passiva à fumaça do cigarro no mesmo protocolo experimental estudado no **Artigo 1**, no cérebro de ratos jovens. Os resultados obtidos demonstraram claramente que a exposição passiva à fumaça do cigarro aumentou os níveis de ácido ascórbico e reduziu a atividade da CAT no cérebro dos ratos jovens. Tendo em vista os resultados obtidos no **Artigos 1** e **3**, notou-se que o dano oxidativo causado nos pulmões (**Artigo 1**) foi mais pronunciado que no cérebro (**Artigo 3**) dos ratos jovens expostos a este protocolo experimental. Esses resultados são pertinentes porque o pulmão é o órgão alvo da fumaça do cigarro e é altamente suscetível à geração de radicais livres. Portanto, propõe-se que a exposição passiva à fumaça de um, dois e três cigarros durante a primeira, segunda e terceira semanas de vida, respectivamente, causou efeitos diferentes nos pulmões e cérebro de ratos jovens, sugerindo um efeito oxidativo dependente do tecido analisado.

Uma vez que os efeitos deletérios causados pela exposição passiva à fumaça do cigarro no primeiro protocolo experimental nos pulmões (**Artigo 1**) e cérebro (**Artigo 3**) de ratos jovens foram discretos, estudou-se o efeito da exposição passiva na qual os animais foram expostos à um maior número de cigarros (**Artigos 2** e **3**) quando comparado com o protocolo experimental anterior (**Artigo 1** e **Artigo 3**).

Os ratos jovens expostos a quatro, cinco e seis cigarros durante a primeira, segunda e terceira semanas de vida, respectivamente, apresentaram dano oxidativo no pulmão (**Artigo 2**), que foi verificado por um aumento na peroxidação lipídica e alteração nas defesas antioxidantes não-enzimáticas (níveis de SHNP e ácido ascórbico) e enzimáticas (atividade da GPx, GR, GST, CAT e SOD). O dano oxidativo no pulmão neste protocolo experimental (**Artigo 2**) foi mais pronunciado que no protocolo experimental anterior (**Artigo 1**), pois verificou-se alterações nas defesas antioxidantes enzimáticas nos pulmões dos ratos jovens expostos à fumaça do cigarro. Utilizando-se este mesmo protocolo experimental (**Artigo 3**) verificou-se que a exposição passiva à fumaça do cigarro causou um estresse oxidativo no cérebro dos ratos jovens demonstrada pelo aumento da peroxidação lipídica, inibição da atividade da δ-ALA-D e redução das defesas antioxidantes enzimáticas (atividade da SOD e CAT) e não-enzimáticas (níveis de SHNP e ácido ascórbico). Portanto, propõe-se que a exposição passiva à fumaça de quatro, cinco e seis cigarros durante a primeira, segunda e terceira semanas de vida, respectivamente, causou efeitos semelhante nos pulmões e cérebro dos ratos jovens. Esses resultados sugerem que com o aumento do insulto, os tecidos (pulmões e cérebro) respondem de maneira semelhante e com isso, ocorrem danos oxidativos similares.

Os efeitos oxidativos das EROs podem ser controlados por antioxidantes exógenos, tais como as vitaminas C e E (Halliwell e Gutteridge, 2000). Alguns estudos têm sugerido um efeito protetor de vitaminas e antioxidantes nas principais doenças relacionadas à fumaça do cigarro (Tiwari, 2004; Anbarasi et al., 2006). Por isso, o conceito que moléculas que contém selênio podem ser melhores nucleófilos (e, portanto, antioxidantes) que antioxidantes clássicos, tem levado a um maior interesse na síntese de compostos orgânicos de selênio (Arteel e Sies, 2001). Entre esses compostos orgânicos de selênio pode-se destacar o $(PhSe)_2$. Esse composto é bastante estudado por nosso grupo de pesquisa e apresenta diversas propriedades farmacológicas já descritas na literatura (Nogueira et al., 2003; Borges et al., 2005; Barbosa et al., 2006; Savegnago et al., 2006, 2007), dentre elas, pode-se destacar o efeito antioxidante em modelos experimentais de estresse oxidativo (Meotti et al., 2004; Santos et al., 2004, 2005a,b; Borges et al., 2008; Luchese et al., 2009; Prigol et al., 2009).

Dessa forma, verificou-se que o tratamento com $(PhSe)_2$ foi efetivo em restaurar o dano oxidativo causado pela exposição à fumaça do cigarro nos pulmões (**Artigos 1** e **2**) e cérebro (**Artigo 3**) dos ratos jovens expostos à fumaça do cigarro nos dois protocolos experimentais. Entretanto, o efeito protetor do $(PhSe)_2$ nos pulmões (**Artigos 1** e **2**) foi mais pronunciado que no cérebro (**Artigo 3**). Isso pode ter ocorrido porque o pulmão tem sido demonstrado como órgão alvo para alguns compostos de selênio em alguns modelos experimentais, já que é metabolizado pelo pulmão (El-Bayoumy, 2001).

Além disso, a terapia com $(PhSe)_2$ aumentou *per se* os níveis de SHNP e ácido ascórbico somente no tecido pulmonar dos ratos jovens, (**Artigos 1** e **2**) e a atividade da GST (**Artigo 2**). Esse efeito do $(PhSe)_2$ não foi verificado no cérebro dos animais tratados somente com o composto (**Artigo 3**). Outros trabalhos do nosso grupo de pesquisa têm demonstrado que o $(PhSe)_2$ aumenta *per se* os níveis de SHNP e ácido ascórbico (Barbosa et al., 2006, 2008; Borges et al., 2008; Luchese et al., 2009; Prigol et al., 2009), o que pode elucidar um dos mecanismos da eficácia antioxidante do $(PhSe)_2$. Os resultados (**Artigo 2**) também reforçam o envolvimento, ao menos em parte, do sistema da glutationa no efeito antioxidante do $(PhSe)_2$.

A atividade antioxidante de vários compostos orgânicos de selênio, tais como o ebselen e o $(PhSe)_2$, parece estar relacionada, ao menos em parte, ao efeito mimético da atividade da GPx (Nogueira et al., 2004; Zhao e Holmgren, 2004). Evidências na literatura indicam que o $(PhSe)_2$ apresenta atividade mimética da GPx, o que pode elucidar um mecanismo pelo qual o composto apresenta efeito antioxidante (Wilson et al., 1989; Meotti et al., 2004). Além disso, Jung et al. (2002) demonstraram que o ebselen além da atividade mimética da GPx, apresenta também atividade mimética da tioltransferase, GST e DHA redutase, que parecem estar relacionadas com o efeito antioxidante deste composto.

Como os mecanismos envolvidos no efeito antioxidante do $(PhSe)_2$ não foram completamente elucidados, realizou-se um estudo (**Manuscrito 1**) com o objetivo de investigar estes mecanismos para esclarecer a bem documentada eficácia deste composto como um potente agente com diversas propriedades farmacológicas. Demonstrou-se (**Manuscrito 1**) que o $(PhSe)_2$ tem atividade mimética da DHA redutase que pode contribuir para a eficácia do composto como um agente antioxidante. A DHA redutase é uma enzima importante que catalisa a redução do DHA a ácido ascórbico (Wells et al., 1995; Xu et al., 1996). Esses resultados ajudariam a explicar o aumento *per se* dos níveis de ácido ascórbico observados no pulmão dos ratos jovens tratados com $(PhSe)_2$ demonstrados nos **Artigos 1** e **2**. Portanto, supõe-se que o aumento do conteúdo de ácido ascórbico encontrado no tratamento

com $(PhSe)_2$, ocorreu devido a atividade mimética da DHA redutase do composto, com isso, aumentando a redução de DHA a ácido ascórbico. A hipótese de que o $(PhSe)_2$ catalisa a reação de redução do DHA a ácido ascórbico é suportada pelos dados apresentados no **Manuscrito 1**, isto é, o $(PhSe)_2$ foi regenerado ao final do ensaio da DHA redutase e detectados por cromatografia gasosa (GC 2010 Shimadzu ®) associada a um sistema detector de ionização em chama. Ou seja, o $(PhSe)_2$ colocado no início do ensaio foi recuperado ao final do ensaio.

No **Manuscrito 1**, verificou-se que o $(PhSe)_2$ apresentou atividade mimética da GST que pode contribuir para o efeito antioxidante do composto, já que, a GST tem sido apontada como uma enzima antioxidante (Mosialou e Morgenstern, 1989; Hayes e Pulford, 1995; Fiander e Schneider, 1999). Com base nos resultados obtidos no **Manuscrito 1** e no **Artigo 2**, sugere-se o envolvimento, ao menos em parte, do sistema da glutationa no efeito antioxidante do $(PhSe)_2$.

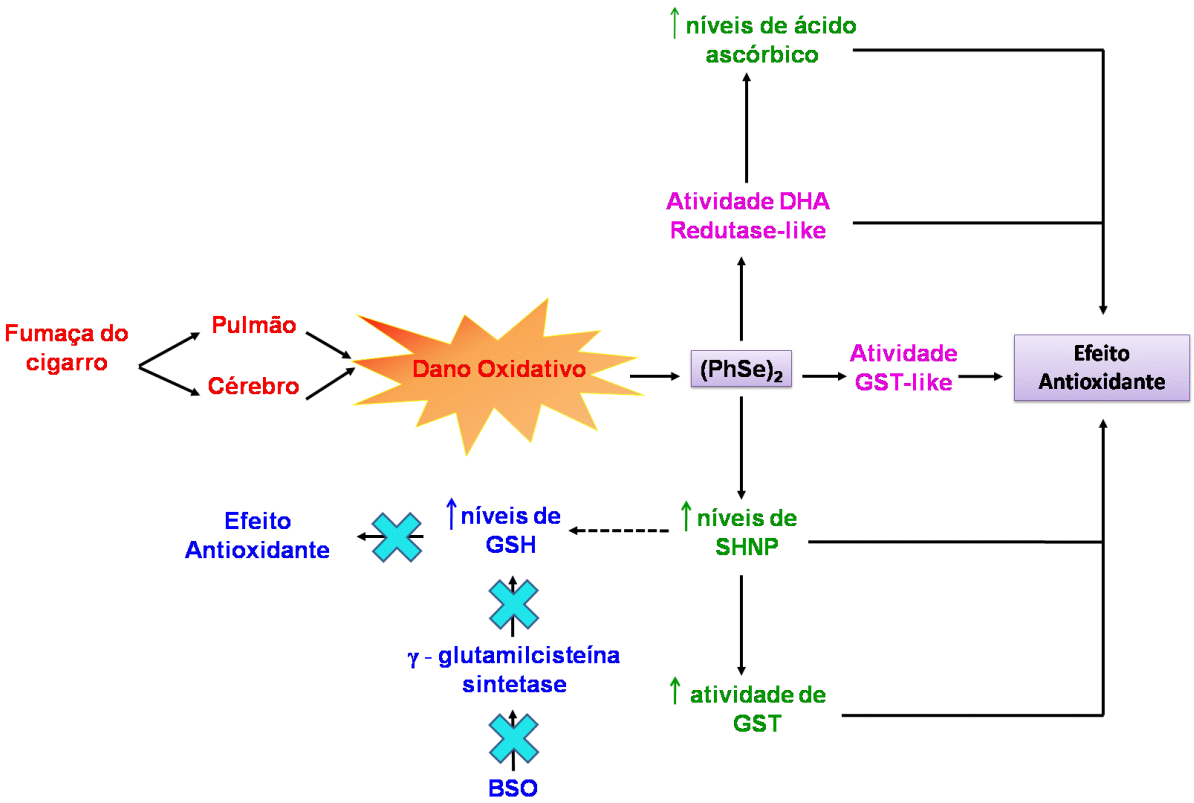
Os resultados do **Manuscrito 1** também demonstraram que a atividade *scavenger* de radicais DPPH e ABTS e a proteção contra a auto-oxidação do Fe^{2+} não estão envolvidas no mecanismo antioxidante deste composto. Alguns compostos orgânicos de selênio e telúrio com efeito antioxidante apresentam efeito scavenger de radicais e protegem contra a auto-oxidação do Fe^{2+} (Acker et al., 2009b; Souza et al., 2009). Entretanto, esses mecanismos antioxidantes não estão relacionados às propriedades farmacológicas do $(PhSe)_2$.

Os resultados dos **Artigos 1** e **2** demonstraram que os animais tratados com $(PhSe)_2$ apresentaram um aumento *per se* nos níveis de SHNP no pulmão. Estes dados nos fizeram questionar se a síntese da glutationa estaria envolvida no efeito antioxidante do composto (**Anexo 1**). Para isso, utilizou-se o BSO, um inibidor da enzima γ-glutamilcisteína sintetase, uma enzima envolvida na síntese de glutationa. Verificou-se que o BSO bloqueou o efeito protetor do $(PhSe)_2$ na redução dos níveis de SHNP provocada pela exposição passiva à fumaça do cigarro no pulmão e fígado de ratos jovens. Com esse resultado supõe-se que a depleção dos níveis de glutationa está envolvida no efeito antioxidante do $(PhSe)_2$, uma vez que, bloqueando a síntese da glutationa, ocorre um bloqueio no efeito antioxidante do composto.

A análise destes 5 trabalhos aqui mencionados permite entender um pouco mais sobre o efeito da exposição passiva à fumaça do cigarro nos pulmões e cérebros de ratos jovens, a propriedade antioxidante do $(PhSe)_2$ e os mecanismos envolvidos nesta ação. Os resultados sugerem que a exposição passiva à fumaça do cigarro causa um estresse oxidativo nos pulmões e cérebro dos ratos jovens. O $(PhSe)_2$ foi efetivo em restaurar os danos causados

pela exposição passiva à fumaça do cigarro nos pulmões e cérebro de ratos jovens nos dois protocolos experimentais. Além disso, o $(PhSe)_2$ apresenta efeito antioxidante por diferentes mecanismos. A ação do $(PhSe)_2$ em aumentar *per se* os níveis de SHNP e ácido ascórbico é um dos mecanismos envolvidos no efeito antioxidante deste composto. Outro mecanismo envolvido no efeito antioxidante do composto é o envolvimento do sistema da glutationa, já que este antioxidante não enzimático é fundamental para o efeito antioxidante do $(PhSe)_2$. É importante salientar que o $(PhSe)_2$ apresenta a atividade mimética da DHA redutase e da GST, demonstrando um novo mecanismo do efeito antioxidante do composto.

No **Esquema 7**, mostrado a seguir, é possível ter uma visão geral dos efeitos da exposição passiva à fumaça do cigarro e do $(PhSe)_2$ além dos mecanismos envolvidos no efeito antioxidante do composto.



Esquema 7 - Visão geral dos mecanismos antioxidantes do $(PhSe)_2$ estudados neste trabalho. As linhas cheias (—) indicam os efeitos demonstrados neste trabalho. A linha pontilhada (---) indica dados baseados na literatura.

6. CONCLUSÕES

De acordo com os resultados obtidos, pode-se concluir que:

- O $(PhSe)_2$ foi efetivo em restaurar o dano oxidativo causado pela fumaça do cigarro no pulmão dos ratos jovens nos dois protocolos experimentais de exposição. Entretanto, o $(PhSe)_2$ restaurou parcialmente o dano oxidativo causado pela exposição passiva à fumaça do cigarro no cérebro dos ratos jovens.
- O aumento *per se* nos níveis das defesas antioxidantes não-enzimáticas (níveis de SHNP e ácido ascórbico) e na atividade da GST nos animais tratados com o $(PhSe)_2$ indica o mecanismo envolvido no efeito antioxidante do composto e o envolvimento, aos menos em parte, neste efeito.
- O $(PhSe)_2$ apresenta atividade mimética da DHA redutase e da GST, sugerindo que esses mecanismos estão envolvidos no efeito antioxidante do composto.
- O $(PhSe)_2$ não apresenta efeito *scavenger* de radicais $DPPH^{\bullet}$ e $ABTS^{\bullet+}$, e não demonstra papel protetor na auto-oxidação do Fe^{2+}.
- A glutationa é fundamental para o efeito antioxidante do $(PhSe)_2$.

7. REFERÊNCIAS

ACKER, C.I., LUCHESE, C., PRIGOL, M., NOGUEIRA, C.W. Antidepressant-like effect of diphenyl diselenide on rats exposed to malathion: involvement of Na+ K+ ATPase activity. Neurosci. Lett. 455, 168-172, 2009a.

ACKER, C.I., BRANDAO, R., ROSARIO, A.R., NOGUEIRA, C.W. Antioxidant effect of alkynylselenoalcohol compounds on liver and brain of rats *in vitro*. doi:10.1016/j.etap.2009.05.002, 2009.

ALBERG A. The influence of cigarette smoking on circulating concentrations of antioxidant micronutrients. **Toxicology** 180, 121–137, 2002.

ALEXI, T.; HUGHES, P.E.; FAULL, R.L.M.; WILLIAMS, C.E. 3-Nitropropionic acid's lethal triplet: cooperative pathway of neurodegeneration. **Neuro. Report.**, R57-R64. 1998.

ANAND, C.V., ANAND, U., AGARWAL, R. Anti-oxidant enzymes, gammaglutamyl transpeptidase and lipid peroxidation in kidney of rats exposed to cigarette smoke. **Indian J. Exp. Biol.** 34, 486– 488, 1996.

ANAND, U. e ANAND, C.V. Anti-oxidant mechanisms in the lungs of pups born to female rats exposed to cigarette smoke. **Med. Sci. Res**, 25, 553 – 554, 1997.

ANBARASI, K., VANI, G., BALAKRISHNA, K., SHYAMALA DEVI, C.S.S. Effect of bacoside A on brain antioxidant status in cigarette smoke exposed rats**. Life Sci.** 78, 1378-1384, 2006.

ANDERSON, H.R. e COOK, D.G. Passive smoking and sudden infant death syndrome: review of the epidemiological evidence. **Thorax** 52, 1003–1009, 1997.

ARDITE, E., PEINADO, V.I., RABINIVICH, R.A., FERNÁNDEZ-CHECA, J.C., ROCA, J., BARBERÀ, J.A. Systemic effects of cigarette smoke exposure in the guinea pig. **Resp. Med.** 100, 1186-1194, 2006.

ARTEEL, G.E., SIES, H. The biochemistry of selenium and the glutathione system. **Environ. Toxicol. Pharmacol.** 10, 153–158, 2001.

BAKER, R.R., PROCTOR, C. Where there's smoke. In: Chemical Education Trust Fund for the Chemical Society and the Royal Institute of Chemistry Royal Society of Chemistry. London: Chemistry in Britain, January, pp. 38–41, 2001.

BANHEGYI, G., BRAUN, L., CSALA, M., PUSKAS, F., MANDL, J. Ascorbate metabolism and its regulation in animals. **Free Radic. Biol. Med.** 23, 793–803, 1997.

BARBOSA, N.B.V., ROCHA, J.B.T., ZENI, G., EMANUELLI, T., BEQUE, M.C., BRAGA, A.L. Effect of organic forms of selenium on δ-Aminolevulinate dehydratase from liver, kidney and brain of adult rats. **Toxicol. Appl. Pharmacol.** 149, 243-253, 1998.

BARBOSA, N.B.V., ROCHA, J.B.T., WONDRACEK, D.C., PEROTONI, J., ZENI, G,, NOGUEIRA, C.W. Diphenyl diselenide reduces temporarily hyperglycemia: possible relationship with oxidative stress. **Chem. Biol. Interact.** 163, 230-238, 2006.

BARBOSA, N.B.V., ROCHA, J.B.T., SOARES, J.C.M., WONDRACEK, D.C., GONÇALVES, J.F., SCHETINGER, M.R.C., NOGUEIRA, C.W. Dietary diphenyl diselenide reduces the STZ-induced toxicity. **Food Chem. Toxicol.** 46, 186–194, 2008.

BASKARAN, S., LAKSHIMI, S., PRASAD, P.R. Effect of cigarette smoke on lipid peroxidation and antioxidant enzymes in albino rat. **Indian J. Exp. Biol.** 37, 1196-1200, 1999.

BEHNE, D., KYRIAKOPOULOS, A. Identification of type I iodothyronine 5'-deiodinase as a selenoenzyme. **Biochem. Biophys. Res. Co.** 173, 1143-1149, 1990.

BENDICH, A., MACHLIN, L.J., SCANDURRA, O., BURTON, G.W., WAYNER, D.D.M. The antioxidant role of vitamin C. **Adv. Free Radic. Biol. Med.** 2, 419 – 444, 1986.

BERGSTEN, P., YU, R., KEHRL, J., LEVINE, M. **Arch. Biochem. Biophys.** 317, 208–214, 1995.

BINDU MP, ANNAMALAI PT. Combined effect of alcohol and cigarette smoke on lipid peroxidation and antioxidant status in rats. **Ind. J. Biochem. Bioph.** 41, 40-44, 2004.

BOGDEN, J.D., KEMP, F.W., BUSE, M., THIND, I.S., LOURIA, D.B., FORGACS, J., LLANOS, G., MONCOYA TERRONES, I. Composition of tobaccos from countries with high and low incidences of lung cancer. I. Selenium, polonium-210, Alternaria, tar, and nicotine. **J Natl Cancer Inst.** 66, 27–31, 1981.

BORGES, L.P., BORGES, V.C., MORO, A.V., NOGUEIRA, C.W., ROCHA, J.B.T., ZENI, G. Protective effect of diphenyl diselenide on acute liver damage induced by 2-nitropropane in rats. **Toxicology** 210, 1–8, 2005.

BORGES, L.P., BRANDAO, R., GODOI, B., NOGUEIRA, C.W., ZENI, G. Oral administration of diphenyl diselenide protects against cadmium-induced liver damage in rats. **Chem.-Biol. Interact.** 171, 15 – 25, 2008.

BRAGA, A.L., SILVEIRA, C.C., ZENI, G., SEVERO, W.A., STEFANI, H.A. Synthesis of selenocetais from enol ethers. **J. Chem. Res.** pp. 206-207. 1996.

BRAGA, A.L., ZENI, G., ANDRADE, L.H., SILVEIRA, C.C. Stereoconservative formation and reativity of α-chalcogen-functionalized vinylithium compounds from bromo-vinylic chalcogens. **Synlett** 5, 595-596. 1997.

BRENNAN, P.A., GREKIN, E.R., MORTENSEN, E.L., MEDNICK, S. Relationship of maternal smoking during pregnancy with criminal arrest and hospitalization for substance abuse in male and female adult offspring. **Am. J. Psychiatry** 159, 48–54, 2002.

BURNS, J. J. Biosynthesis of L-ascorbic acid: basic defect in scurvy. **Am. J. Med.** 26, 740 – 748, 1959.

BURRI, P.H. The postnatal growth of the rat lung. **Morphology Anat. Rec.** 180, 77–98, 1974.

BURRI, P.H. Structural aspects of postnatal lung development-alveolar formation and growth. **Biol. Neonate** 89, 313–322, 2006.

CASALINO, E., SBLANO, C., LANDRISCINA, V., CALZARETTI, G., LANDRISCINA, C. Rat liver glutathione Stransferase activity stimulation following acute cadmium or manganese intoxication. **Toxicology**, 200, 29-38. 2004.

CERVELLO, I., LAFUENTE, A., GIRALT, M., MALLOL, J. Enhanced glutathione S-transferase (GST) activity in pregnantrats treated with benzo(a)pyrene. Placenta 13, 273 – 280, 1992.

CHASSEAUND, L.F. The role of glutathione and glutathione S-transferase in the chemicals carcinogens and other electrophilic agents. **Adv. Cancer Res.** 29, 175 – 274, 1979.

CHURCH, D.F. e PRYOR, W.A. Free radicals chemistry of cigarette smoke and its toxicological implications. **Environ. Health Persp.** 64, 111-126, 1985.

COMASSETO, J.V. Vinylic selenides. **J. Organomet Chem.** v. 253, p. 131-181, 1983.

COOK, D.G., STRACHAN, D.P., CAREY, I.M. Health effects of passive smoking. Parental smoking and spirometric indices in children. **Thorax** 53, 884–893, 1998.

CROSS CE, O'NEILL CA, REZNICK AZ, HU ML, MARCOCCI L, PACKER L, FREI B. Cigarette smoke oxidation of human plasma constituents. **Ann. NY Acad. Sci.** 686, 72-90, 1993.

CROSS, C.E., VALACCHI, G., SCHOCK, B., WILSON, M., WEBER, S., EISERICH, J., VAN DER VLIET, A. Environmental oxidant pollutant effects on biologic systems. **Am. J. Respir. Crit. Care Med.** 166, S44 – S50, 2002.

DAVIES, K.J.A. Oxidative damage and repair: Chemical, biological and medical aspects. Oxford: Pergamon, pp. 910, 1991.

DAWSON, V.L. e DAWSON, T.M. Free radicals and neuronal cell death. **Cell. Death and Differentiation**, 3, 71-78. 1996.

DEJIN-KARLSSON, E., HANSON, B.S., OSTERGREN, P.O., SJOBERG, N.O., MARSAL, K. Does passive smoking in early pregnancy increase the risk of small-for-gestational-age infants? **Am. J. Public Health** 88, 1523–1527, 1998.

DENNERY, P.A. Effects of oxidative stress on embryonic development. Birth **Defects Res. C: Embryo. Today** 81, 155–162, 2007.

DEZATEUX, C. e STOCKS, J. Lung development and early origins of childhood respiratory illness. **Br. Med. Bull.** 53, 40–57, 1997.

DEZATEUX, C., STOCKS, J., DUNDAS, I., FLETCHER, M.E. Impaired airway function and wheezing in infancy: the influence of maternal smoking and a genetic predisposition to asthma. **Am. J. Respir. Crit. Care Med.** 159, 403–410, 1999.

DIXON, S. J., e WILSON, J. X. **J. Bone Miner. Res.** 7, 675–681, 1992.

DROGE, W. Free radicals in the physiological control of cell function. **Physiol. Rev.** 82, 47-95, 2002.

DUMONT, E.; VANHAECKE, F.; CORNELIS, R. Selenium speciation from food source to metabolites: a critical review. **Anall. Bioanal. Chem.** 385, 1304-1343, 2006.

EISERICH, J.P., VLIET, A.V., HANDELMAN, G.J., HALLIWELL, B., CROSS, C.E. Dietary antioxidants and cigarette smoke-induced biomolecular damage: a complex interaction. **Am. J. Clin. Nutr.** 62, 1490–1500, 1995.

EL-BAYOUMY, K. The protective role of selenium on genetic damage and on cancer. Mutat. Res. 475, 123–139, 2001.

ESKENAZI, B. e CASTORINA, R. Association of prenatal maternal or postnatal child environmental tobacco smoke exposure and neurodevelopmental and behavioral problems in children. **Environ. Health Perspect.** 107, 991–1000, 1999.

FLOREK E, MARSZALEK, A., BICZYSKO, W., SZYMANOWSKI, K. The experimental investigations of the toxic influence of tobacco smoke affecting progeny during pregnancy. **Hum. Exp. Toxicol.** 18, 245 – 251, 1999.

FORONJY, R., D'ARMIENTO, J. The effect of cigarette smoke-derived oxidants on the inflammatory response of the lung. **Clin. Appl. Immunol. Rev.** 6, 53-72, 2006.

FOYER, C.H.; LELANDAIS, M.; KUNERT, K.J. Photooxidative stress in plants. **Physiol. Plant** 92, 696-717, 1994.

FIANDER, H., SCHNEIDER, H. Compounds that induce isoforms of glutathione S-transferase with properties of a critical enzyme in defense against oxidative stress. **Biochem. Biophys. Res. Commun.** 262, 591 – 595, 1999.

FRIDOVICH, I. Superoxide dismutases - an adaptation to a paramagnetic gas. J**. Biol. Chem.** 264, 7761 – 7764, 1989.

GIANNI, P.; JAN, K.J.; DOUGLAS, M.J.; STUART, P.M.; TARNOPOLSKY, M.A. Oxidative stress and the mitochondrial theory of aging in human skeletal muscle. **Exp. Geront.** 39, 1391-1400, 2004.

GIDDING, S.; MORGAN, W.; PERRY, C.; ISABEL-JONES, J.; BRICKER, J.T. Active and passive tobacco exposure: a serious pediatric health problem. A statement from the Committee on Atherosclerosis and Hypertension in Children, Council on Cardiovascular disease in the young, American Hearth Association. **Circulation** 90, 2581 – 2590, 1994.

GHISLENI, G., PORCIÚNCULA L.O., CIMAROSTI, H., ROCHA, J.B.T., SALBEGO, C.G., SOUZA, D.O. Diphenyl diselenide protects rat hippocampal slices submitted to oxygen–glucose deprivation and diminishes inducible nitric oxide synthase immunocontent. **Brain. Res.** 986, 196-199, 2003.

GREEN, C.R. e RODGMAN, A. The tobacco chemists research conference: a half century forum for advances in analytical methodology of tobacco and its products. **Rec. Adv. Tob. Sci.** 22, 131–304, 1996.

GUNTHEROTH, W.G. E SPIERS, P.S. Sleeping prone and the risk of sudden infant death syndrome. **JAMA** 267, 2359-2362, 1992.

GUELCIN, I. Antioxidant activity of L-adrenaline: A structure-activity insight. **Chem.-Biol. Interact.** 179, 71 – 80, 2009

HAFSTROM, O., MILERAD, J., ASOKAN, N., POOLE, S.D., SUNDELL, H.W. Nicotine delays arousal during hypoxemia in lambs. **Pediatr. Res.** 47, 646–652, 2000.

HALLIWELL, B., GUTTERIDGE, J.M.C. Role of free-radicals and catalytic metal-ions in human-disease – an overview. **Meth. Enzymol.** 186, 1 – 85, 1990.

HALLIWELL, B. Reactive oxygen species and the central nervous system. **J. Neurochem.**, 59, 1609-1623, 1992.

HALLIWELL, B., GUTTERIDGE, J.M.C. Free radicals in Biology and medicine. 3rd ed. Oxford: Oxford Science Publications, 2000.

HALLIWELL B, GUTTERIDGE JMC. Free radicals in biology and medicine. 4th ed. Oxford/UK: Clarendon Press/Oxford Science Publications; 2007.

HANRAHAN, J.P., TAGER, I.B., SEGAL, M.R., TOSTESON, T.D., CASTILE, R.G., VAN VUNAKIS, H., WEISS, S.T., SPEIZER, F.E. The effect of maternal smoking during pregnancy on early infant lung function. **Am. Rev. Respir. Dis.**145, 1129–1135, 1992.

HAYES, J.D., PULFORD, D.J. The glutathione *S*-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. **Crit. Rev. Biochem. Mol. Biol.** 30, 445-600, 1995.

HECHT, S.S. Tobacco smoke carcinogens and lung cancer. **J. Natl. Cancer Inst.** 91, 1194–1210, 1991.

HIPPELI, S., ELSTNER, E.F. Transition metal ion-catalyzed oxygen activation during pathogenic processes. **FEBS Lett.** 443, 1–7, 1999.

HO, Y.S. Transgenic and knockout models for studying the role of lung antioxidant enzymes in defense against hyperoxia. **Am. J. Respir. Crit. Care Med.** 166, S51 – S56, 2002.

HOFFMAN, J.L. e MCCONELL, K.P. Periodate-oxidized adenosine inhibits the formation of dimethylselenide and trimethylselenium ion in mice treated with selenite. **Arch. Biochem. Biophys.** 254, 534-540, 1986.

HOFHUIS, W., DE JONGSTE, J.C., MERKUS, P.J. Adverse health effects of prenatal and postnatal tobacco smoke exposure on children. **Arch. Dis. Child.** 88, 1086–1090, 2003.

HOLMGREN, A. Thioredoxin. **Annu. Rev. Biochem.** 54, 237-271, 1985.

HOO, A.F., HENSCHEN, M., DEZATEUX, C., COSTELOE, K., STOCKS, J. Respiratory function among preterm infants whose mothers smoked during pregnancy. **Am. J. Respir. Crit. Care. Med.** 158, 700–705, 1998.

HOWARD, D.J., OTA, R.B., BRIGGS, L.A., HAMPTON, B., PRITSOS, C.A. Oxidative stress induced by environmental tobacco smoke in the workplace is mitigated by antioxidant supplementation. **Cancer Epidem. Biomar. Prev.** 7, 981 – 988, 1998.

INTERNATIONAL AGENCY FOR RESEARCH ON CANCER (IARC). Biological Data Relevant to the Evaluation of Carcinogenic Risk to Humans. Tobacco smoking. Carcinogenicity studies in Animals. IARC Monographs on the Evaluation of the Carcinogenic Risks of Chemicals to Humans. Lyon. 38, 127–139, 1992.

JANOFF, A., PRYOR, W.A., BENGALI, Z.H. Effects of tobacco smoke components on cellular and biochemical processes in the lungs. **Am. Rev. Respir. Dis.** 136, 1058-1064, 1987.

JEFFERY, P.K. Effects of cigarette smoke and air pollutants on the lower respiratory tract. In: Holgate ST, Samet JM, Koren HS, Maynard RL, editors. Air pollution and health. Academic Press. pp. 219–268, 1999.

JOSEPHY, P. D. Molecular Toxicology, New York: Oxford University Press, 1997.

JUNG, C.H., WASHBURN, M.P., WELLS. W.W. Ebselen has dehydroascorbate reductase and thioltransferase-like activities. **Biochem. Biophys. Res. Com.** 291, 550 – 553. 2002.

KHOMENKO, T., SZABO, S., DENG, X., ISHIKAWA, H., ANDERSON, G., MCLAREN, G. Role of iron in the pathogenesis of cysteamine-induced duodenal ulceration in rats. **Am. J. Physiol.-Gastr. L.** 296, G1277-G1286, 2009.

KLAUNIG, J.E. e KAMENDULIS, L.M. The role of oxidative stress in carcinogenesis. **Ann. Rev. Pharmacol. Toxicol.** 44, 239–267, 2004.

KLAYMAN, D.L., GÜNTHER, W.H. (eds.). **Organic selenium compounds: their chemistry and biology**. New York: John Wiley and sons, 68-157, 1973.

KLONOFF-COHEN, H.S.; EDELSTEIN, S.L.; LEFKOWITZ, E.S. The effect of passive smoking and tobacco exposure through breast milk on sudden infant death syndrome. **JAMA** 273, 795 – 798, 1995.

KOKSAL, E., GULCIN, I. Antioxidant activity of cauliflower (*Brassica oleracea L.*). **Turk. J. Agric. For.** 32, 65 – 78, 2008

KOKSAL, E., GULCIN, I., BEYZA, S., SARIKAYA, O. BURSAL, E. In vitro antioxidant activity of silymarin. **J. Enzym. Inhib. Med. Ch.** 24, 395 – 405, 2009.

KOSANOVIC, M. e JOKANOVIC, M. The association of exposure to cadmium through cigarette smoke with pregnancy-induced hypertension in a selenium deficient population. **Environ. Toxicol. Phar.** 24, 72 – 78, 2007

KOUL A., BHATIA, V., BANSAL, M.P. Effect of alpha-tocopherol on pulmonary antioxidant defense system and lipid peroxidation in cigarette smoke inhaling mice. **BMC Biochemistry** 2, 14–18, 2001.

KRIZ, P., BOBAK, M., KRIZ, B. Parental smoking, socioeconomic factors, and risk of invasive meningococcal disease in children: a population based case-control study. **Arch. Dis. Child** 83, 117–121, 2000.

LEVINE, M. New concepts in the biology and biochemistry of ascorbic acid. **N. Engl. J. Med.** 314, 892–902, 1986.

LUCHESE, C., PINTON, P., NOGUEIRA, C.W. Brain and lungs of rats are differently affected by cigarette smoke exposure: antioxidant effect of an organoselenium compound. **Pharmacol. Res.** 59, 194 – 201, 2009.

MARTIN, J.L. e GERLACK, M.L. Selenium metabolism in animals. **Ann. NY Acad. Sci.** 192, 193-199, 1972.

MCMARTIN, K.I., PLATT, M.S., HACKMAN, R., KLEIN, J.; SMIALEK, J.E.; VIGORITO, R.; KOREN, G. Lung tissue concentrations of nicotine in sudden infant death syndrome (SIDS). **J. Pediatr.** 140, 205–209, 2002.

MEISTER, A. Glutathione-ascorbic acid antioxidant system inanimals. **J. Biol. Chem.** 269, 9397–9400, 1994.

MEOTTI, F.C., STANGHERLIN, E.C., ZENI, G., NOGUEIRA, C.W., ROCHA, J.B.T. Protective role of aryl and alkyl diselenides on lipid peroxidation. **Environ. Res.** 94, 276-282, 2004.

MILERAD, J. e SUNDELL, H. Nicotine exposure and the risk of SIDS. **Acta Paediatr. Suppl.** 82, 70–72, 1993.

MILLER, M., AUST, S.D. Studies of ascorbate-dependent, iron-catalyzed lipid peroxidation, **Arch. Biochem. Biophys.** 271, 113 – 119, 1989.

MORCILLO EJ, ESTRELA J, CORTIJO J. Oxidative stress and pulmonary inflammation: pharmacological intervention with antioxidants. **Pharmacol. Res.** 40, 393–404, 1999.

MORGANE, P.J., MOKLER, D.J., GALLER, J.R. Effects of prenatal protein malnutrition on the hippocampal formation. **Neurosci. Biobehav. Rev.** 26, 471-483, 2002.

MOSIALOU, E., MORGENSTERN, R. Activity of rat liver microsomal glutathione transferase toward products of lipid peroxidation and studies of the effect of inhibitors on

glutathione-dependent protection against lipid peroxidation. **Arch. Biochem. Biophys.** 275, 289 – 294, 1989.

MOZIER, N.M., MCCONELL, K.P., HOFFMAN, J.L. S-adenosyl-L-methionine: thioeter S-methyltransferase, a new enzyme in sulfur and selenium metabolism. **J. Biol. Chem.** 263, 4527-4531, 1988.

NAVARRO-ALARCÓN, M.; LÓPEZ-MARTINEZ, M.C. Essentiality of selenium in the human body: relationship with different deseases. **Sci. Tot. Environ.** 249, 347-371, 2000.

NOGUEIRA, C.W., QUINHONES, E.B., JUNG., E.A.C., ZENI, G., ROCHA, J.B.T. Anti-inflammatory and antinociceptive activity of diphenyl diselenide. **Inflamm. Res.** 52, 56 – 63, 2003.

NOGUEIRA, C.W., ZENI, G., ROCHA, J.B.T. Organoselenium and organotellurium compounds: toxicology and pharmacology. **Chem. Rev.** 104, 6255– 6286, 2004.

OLDFIELD, J.E. The Two faces of selenium. **J. Nutr.** 117, 2002-2008, 1987.

OZAN, E., SONMEZ, M. F., OZAN, S., COLAKOGLU, N., YILMAZ, S., KULOGLU, T. Effects of melatonin and vitamin C on cigarette smoke-induced damage in the kidney. Toxicol. Ind. Health 25, 479 – 485, 2007.

OZKAN A, FISKIN K, AYHAN AG. Effect of vitamin E and selenium on antioxidant enzymes in brain, kidney and liver of cigarette smoke-exposed mice. **Biologia** 62, 360-364, 2007.

PADMAJA, S., HUIE, R.E. The reaction of nitric oxide with organic peroxyl radicals. **Biochem. Biophys. Res. Commun.** 195, 539-544, 1993.

PANDA K, CHATTOPADHYAY R, GHISH MK, CHATTOPADHYAY DJ, CHATTERJEE IB. Vitamin C prevents cigarette smoke-induced oxidative damage of proteins and increased proteolysis. **Free Radic. Biol. Med.** 27, 1064-1079, 1999.

PARK, E.M., PARK, Y.M., GWAK, Y.S. Oxidative damage in tissues of rats exposed to cigarette smoke. **Free Radic. Biol. Med.** 25, 79 – 86, 1998.

PARNHAM, M.J. e GRAF, E. Pharmacology of synthetic organic selenium compounds. **Prog. Drug Res.** 36, 10-47, 1991.

PASUPATHI, P., BAKTHAVATHSALAM, G., RAO Y.Y., FAROOK, J. Cigarette smoking-Effect of metabolic health risk: A review. **Diabetes Metab. Syn.: Clin. Res. Rev.** 3, 120–127, 2009.

PAULMIER, C. Selenium reagents and intermediates. In: **Organic Synthesis**. Oxford: Pergamon. 1986.

PERFETTI, T.A., COLEMAN, W.M., SMITH, W.S. Determination of mainstream and sidestream cigarette smoke components for cigarettes of different tobacco types and a set of reference cigarettes. **Beitr. Tabakforsch. Int.** 18, 95–113, 1998.

PETRAGNANI, N., RODRIGUES, R., COMASSETO, J.V. **Organomet. Chem.** 114-281, 1976.

POULI, E.A., HATZINIKOLAOU, G.D., PIPERI, C., STAVRIDOU, A., PSALLIDOPOULOS, C.M., STAVRIDIS, C.J. The cytotoxic effect of volatile organic compounds of the gas phase of cigarette smoke on lung epithelial cells. **Free Radic. Biol. Med.** 34, 345–355, 2003.

POWER, J.H.T., ASAD, S., CHATAWAY, T.K., CHEGINI, F., MANAVIS, J., TEMLETT, J.A., JENSEN, P.H., BLUMBERGS, P.C., GAI, W. Peroxiredoxin 6 in human brain: molecular forms, cellular distribution and association with Alzheimer's disease pathology. **Acta Neuropathol.** 115, 611–622, 2008.

PRIGOL, M., LUCHESE, C., NOGUEIRA, C.W. Antioxidant effect of diphenyl diselenide on oxidative stress caused by acute physical exercise in skeletal muscle and lungs of mice. **Cell Biochem. Funct.** DOI:10.1002/cbf.1559, 2009a.

PRIGOL, M., SCHUMACHER, R.F., NOGUEIRA, C.W., ZENI, G. Convulsant effect of diphenyl diselenide in rats and mice and its relationship to plasma levels. **Toxicol. Lett.** DOI:10.1016/j.toxlet.2009.04.026, 2009b.

PRYOR, W.A., HALE, B.J., PREMOVIC, P.I., CHURCH, D.F. The radicals in cigarette tar: their nature and suggested physiological implications. **Science** 220, 425–7, 1983.

PRYOR, W.A. e STONE, K. Oxidants in cigarette smoke. Radicals, hydrogen peroxido, peroxynitrate e peroxynitrite. **Ann. NY Acad. Sci.** 686, 12–27, 1993.

PUNTEL, G.O., GUBERT, P., PERES, G.L., BRESOLIN, L., ROCHA, J.B.T, PEREIRA, M.E., CARRATU, V.S., SOARES, F.A.A. Antioxidant properties of oxime 3-(phenylhydrazono) butan-2-one. **Arch. Toxicol.** 82, 755 – 762, 2008.

PUNTEL, G.O., DE CARVALHO, N.R., GUBERT, P., PALMA, A.S., CORTE, C.L.D., AVILA, D.S., PEREIRA, M.E., CARRATU, V.S., BRESOLIN, L., DA ROCHA, J.B.T, SOARES, F.A.A. Butane-2,3-dionethiosemicarbazone: An oxime with antioxidant properties. **Chem.-Biol. Interact.** 177, 153 – 160, 2009.

REDDY, S., FINKELSTEIN, E.I., WONG, P., PHUNG, A., CROSS, C.E., VAN DER VLIET, A. Identification of glutathione modifications by cigarette smoke. **Free Radic. Biol. Med.** 33, 1490-1498, 2002.

VERA, J. C., RIVAS, C. I., ZHANG, R. H., FARBER, C. M., GOLDE, D.W. **Blood** 84, 1628–1634, 1994.

RIVAS, C.I., ZUNIGA, F.A., SALAS-BURGOS, A., MARDONES, L., ORMAZABAL, V., VERA, J. C. Vitamin C transporters. **J. Physiol. Biochem.** 64, 357 – 375, 2008.

ROSSATO, J.I., KTZER, L.A., CENTURIÃO, F.B., SILVA, S.J.N., LUDTKE, D.S., ZENI, G., BRAGA, A.L., RUBIN, M.A., ROCHA, J.B.T. Antioxidant propeties of new chalcogenides against lipid peroxidation in rat brain. **Neurochem. Res.** 27, 297-303, 2002.

RUMSEY, S.C., KWON, O., XU, G.W., BURANT, C.F. SIMPSON I., LEVINE, M. Glucose Transporter Isoforms GLUT1 and GLUT3 Transport Dehydroascorbic Acid. **J. Biol. Chem.** 272, 18982 – 18989, 1997.

SANTOS, F.W.; ORO, T.; ZENI, G.; ROCHA, J.B.T.; NASCIMENTO, P.C.; NOGUEIRA, C.W. Cadmium induced testicular damage and its response to administration of succimer and diphenyl diselenide in mice. **Toxicol. Lett.** 152, 255– 263, 2004.

SANTOS, F.W., ZENI, G., ROCHA, J.B.T., DO NASCIMENTO, P.C., MARQUES, M.S., NOGUEIRA, C.W. Efficacy of 2,3-dimercapto-1-propanesulfonic acid (DMPS) and diphenyl diselenide on cadmium induced testicular damage in mice. **Food Chem. Toxicol.** 43, 1723 – 1730, 2005a.

SANTOS, F.W., ZENI, G., ROCHA, J.B.T., WEIS, S.N., FACHINETTO, J.M., FAVERO, A.M., NOGUEIRA, C.W. Diphenyl diselenide reverses cadmium-induced oxidative damage on mice tissues **Chem.-Biol. Interact.** 151, 159 – 165, 2005b.

SAVEGNAGO, L., TREVISAN, M., ALVES, D., ROCHA, J.B.T., NOGUEIRA, C.W., ZENI, G. Antisecretory and antiulcer effects of diphenyl diselenide. **Environ. Toxicol. Pharmacol.** 21, 86–92, 2006.

SAVEGNAGO, L., PINTO, L.G., JESSE, C.R., ALVES, D., ROCHA,, J.B.T., NOGUEIRA, C.W., ZENI, G. Antinociceptive properties of diphenyl diselenide: Evidences for the mechanism of action. **Eur. J. Pharmacol.** 555, 129-38, 2007.

SAVEGNAGO, L., JESSE, C.R., PINTO, L.G., ROCHA, J.B.T., BARANCELLI, D.A., NOGUEIRA, C.W., ZENI, G. Diphenyl diselenide exerts antidepressant-like and anxiolytic-like effects in mice: Involvement of L-arginine-nitric oxide-soluble guanylate cyclase pathway in its antidepressant-like action. **Pharmacol. Biochem. Behav.** 88, 418-426, 2008.

SCHWARTZ, K.; FOLTZ, P. J. Selenium as a integral part of facto 3 against dietary necrotic liver degeneration. **J. Am. Chem. Soc.** 79, 200-214, 1957.

SIES, H. Oxidative stress: oxidants and antioxidants. **Exp. Physiol.** 82, 291-295. 1997.

SMITH, C.J., PERFETTI, T.A., RUMPLE, M.A., RODGMAN, A., DOOLITTLE, D.J. "IARC group 3A carcinogens" reported in cigarette mainstream smoke. **Food Chem. Toxicol.** 38, 371–383, 2000.

SOARES, J.R., DINS, T.C.P., CUNHA, A.P., AMEIDA LM. Antioxidant activity of some extracts of *Thymus zygis*. **Free Radic. Res.** 26, 469–478, 1997.

SOUZA, A.C.G, LUCHESE, C., NETO, J.S.S, NOGUEIRA, C.W. Antioxidant effect of a novel class of telluroacetilene compounds: Studies in vitro and in vivo. **Life Sci.** 84, 351 – 357, 2009.

STADTMAN, T. C. Selenium-dependent enzymes. **Annu. Rev. Biochem.** 49, 93-110, 1980.

STICK, S.M., BURTON, P.R., GURRIN, L., SLY, P.D., LESOUËF, P.N. Effects of maternal smoking during pregnancy and a family history of asthma on respiratory function in newborn infants. **Lancet** 348, 1060–1064, 1996.

STRACHAN, D.P. e COOK, D.G. Health effects of passive smoking. Parental smoking and lower respiratory illness in infancy and early childhood. **Thorax** 52, 905–914, 1997.

STRACHAN, D.P. e COOK, D.G. Health effects of passive smoking. Parental smoking and childhood asthma: longitudinal and case-control studies. **Thorax** 53, 204–212, 1998a.

STRACHAN, D.P. e COOK, D.G. Health effects of passive smoking. Parental smoking and allergic sensitisation in children. **Thorax** 53, 117–123, 1998b.

SUBRAMANIAM, S., SRINIVASAN, S., BUMMER, P.M., GAIROLA, C.G. Perinatal sidestream cigarette smoke exposure and the developing pulmonary surfactant system in rats. **Hum. Exp. Toxicol.** 18, 206 – 211, 1999.

SUZUKI, K.T., SOMEKAWA, L., SUZUKI, N. Distribution and reuse of 76Se-selenosugar in selenium-deficient rats. **Toxicol. Appl. Pharmacol.** 216, 303-308, 2006.

TAO, F., GONZALEZ-FLECHA, B., KOBZIK, L. Reactive oxygen species in pulmonary Inflammation by ambient particulates. **Free Radic. Biol. Med.** 35, 327–340, 2003.

TAGER, I.B., NGO, L., HANRAHAN, J.P. Maternal smoking during pregnancy. Effects on lung function during the first 18 months of life. **Am. J. Respir. Crit. Care Med.** 152, 977–983, 1995.

TAYLOR, A.E., JOHSON, D.C., KAZEMI, H. Environmental tobacco smoke and cardiovascular disease: a position paper from the Council on Cardiopulmonary and Critical Care, American Heart Association. **Circulation** 86, 1 – 4, 1992.

THORN, N.A., NIELSEN, F.S., JEPPESEN, C. K. **Acta Physiol. Scand.** 141, 97–106, 1991.

TIMBRELL, J. Principles of biochemical toxicology, 3ª ed, London: Taylor & Francis, 2000.

TIWARI, A.K. Antioxidants: new-generation therapeutic base for treatment of polygenic disorders. **Curr. Sci.** 86, 1092-1102, 2004.

TOLBERT, B.M. Metabolism and function of ascorbic acid and its metabolites. **Int. J. Vitam. Nutr. Res. Suppl.** 27, 121 – 138, 1985.

TSCHANZ, S.A., MAKANYA, A.N., HAENNI, B., BURRI, P.H. Effects of neonatal high-dose short-term glucocorticoid treatment on the lung: a morphologic and morphometric study in the rat. **Pediatr. Res.** 53, 72–80, 2003.

AK, T., GULCIN, I. Antioxidant and radical scavenging properties of curcumin. **Chem.-Biol. Interact**. 174, 27–37, 2008.

TUTKA, P., WIELOSZ, M., ZATONSKI, W. Exposure to environmental tobacco smoke and children health. **Int. J. Occup. Med. Environ. Health** 15, 325-335, 2002.

URSINI, F.; HEIM, S.; KIESS, M.; MAIORINO, M.; ROVERI, A.; WISSING, J.; FLOHÉ, L. Dual function of the seleno-protein PHGPx during sperm maturation. **Science** 285, 1393-1396, 1990.

VALENCA, S.S., BEZERRA, F.S., ROMANA-SOUZA, B., PAIVA, R.O., ALTO COSTA, A.M., PORTO, L.C. Supplementation with vitamins C and E improves mouse lung repair. **J. Nutr. Biochem.** 19, 604 – 611, 2008.

VALENTINE, J.L., KANG, H.K., SPIVEY, G.H. Selenium levels in human blood, urine and hair in response to exposure via drinking water. **Environ. Res.** 17, 347-355, 1978.

VAN BINH, P.N., ZHOU, D., BAUDOUIN, F., MARTIN, C., RADIONOFF, M., DUTERTRE, H., MARCHAND, V., THEVENIN, M., WARNET, J.M., DUC, H.T. Modulation of the primary and the secondary antibody response by tobacco smoke condensates. **Biomed. Pharmacother.** 58, 527 – 530, 2004a.

VAN BINH, P.N., ZHOU, D., BAUDOUIN, F., MARTIN, C., RADIONOFF, M., DUTERTRE, H., MARCHAND, V., THEVENIN, M., WARNET, J.M., DUC, H.T. In vitro and in vivo immunotoxic and immunomodulatory effects of nonsupplemented and selenium-supplemented cigarette smoke condensate. **Biomed. Pharmacother.** 58, 90 – 94, 2004b.

VAN DER VLIET A, CROSS CE. Oxidants, nitrosants, and the lung. **Am. J. Med.** 109, 398–421, 2000.

XIA, L., CRANE-GODREAU, M., LEITERA, J.C., BARTLETT D. Gestational cigarette smoke exposure and hyperthermic enhancement of laryngeal chemoreflex in rat pups. **Resp. Physiol. Neurobi.** 165, 161 – 166, 2009.

WELCH, R.W., BERGSTEN, P., BUTLER, J.D., LEVINE. **M. Biochem. J.** 294, 505–510, 1993.

WELCH, R.W., WANG, Y., CROSSMAN, A.J., PARK, J.B., KIRK, K.L., LEVINE, M. J. **Biol. Chem.** 270, 12584–12592, 1995.

WELLS, W.W., XU, D.P., WASHBURN, M.P. Glutathione – dehydroascorbate oxidoreductases. **Methods Enzymol.** 252, 30 – 38, 1995.

WHANGER, P.D., PEDERSEN, N.D., HATFIELD, J., WESWING, P.H. Absorpion of selenite and selenomethionine from ligated digestive tract segments in rats. **Proc. Soc. Exp. Biol. Med.** 153, 295-297, 1976.

WILBER, C.G. Toxicology of selenium: A review. **Clin. Toxicol.** 17, 171-230, 1980.

WILSON, S.R., ZUCKER, P.A., HUANG, R.C., SPECTOR, A. Development of synthetic compounds with glutathione peroxidase activity. **J. Am. Chem. Soc.** 111, 5936, 1989.

WILSON, J.X. The physiological role of dehydroascorbic acid. **FEBS Lett.** 527, 5 – 9, 2002.

WINGLER, K., BRIGELIUS-FLOHÉ, R. Gastrointestinal glutathione peroxidase. **Biofactors** 10, 245-249, 1999.

WINKLER, B. S., ORSELLI, S. M., REX, T. S. The redox couple betweenglutathione and ascorbic acid: a chemical and physiologicalperspective. **Free Radic. Biol. Med.** 17, 333–349, 1994.

WITSCHI, H., ESPIRITU, I., MARONPOT, R.R., PINKERTON, E.K., JONES, D. The carcinogenic potential of the gas phase of tobacco smoke. **Carcinogenesis** 18, 2035–2042, 1977.

WORLD HEALTH ORGANIZATION (WHO). Environmental Health Criteria, Cadmium. 1st ed. **World Health Organization**, Geneva, Switzerland, vol. 134. 1992.

WORLD HEALTH ORGANIZATION (WHO). Tobacco free initiative. **International consolation on environmental tobacco smoke and child health report.** Geneva, 1999.

XU, D.P. WASHBURN, M.P. SUN, G.P. WELLS, W.W. Purification and characterization of a glutathione dependent dehydroascorbate reductase from human erythrocytes. **Biochem. Biophys. Res. Commun.** 221, 117 – 121, 1996.

YILMAZ S, BENZER F, OZAN S, GURGOZE SY. Oxidative damage and arginase activity in tissues of rats exposed to cigarette smoke. **Rev. Med. Vet.** 159, 79-86, 2008.

YOKOYAMA, E. Ventilatory functions of normal rats of different ages. **Comp. Biochem. Physiol. A** 75, 77–80, 1983.

ZHAO, R., HOLMGREN, A. Ebselen is a dehydroascorbate reductase mimic, facilitating the recycling of ascorbate via mammalian thioredoxin systems. **Antioxid. Redox. Signal.** 6, 99 – 104, 2004.

ANEXOS

8. Anexos

8.1. Anexo A: Resultados complementares

8.1.1.Objetivo:

Verificar o envolvimento da depleção dos níveis de glutationa no efeito antioxidante do $(PhSe)_2$ no dano oxidativo causado pela exposição passiva à fumaça do cigarro nos pulmões e fígado de ratos jovens. Para isso foi utilizado a L-Butionina (S,R) Sulfoximina (BSO), que é um composto que depleta os níveis de glutationa intracelular. Este composto exerce sua ação por inibir irreversivelmente a enzima γ-glutamil cisteína sintase.

8.1.2. Materiais e Métodos:

Animais: Foram utilizados ratos Wistar bebês de ambos os sexos de 20 dias de idade, provenientes do Biotério Central da Universidade Federal de Santa Maria. Os experimentos foram conduzidos de acordo com as normas do Comitê de Ética e Bem-Estar Animal da Universidade Federal de Santa Maria.

Materiais: Foram utilizados cigarros de diversas marcas comerciais com as concentrações de nicotina de 0,8 mg. O $(PhSe)_2$ foi sintetizado de acordo com a literatura (Paulmier, 1986).

8.1.2.1. Protocolo Experimental:

Os animais foram divididos nos seguintes grupos:

Grupo Controle: os animais receberam óleo de canola por via oral (v.o.), e salina pela via intraperitoneal (i.p.) e não foram expostos à fumaça do cigarro.

Grupo $(PhSe)_2$: os animais receberam $(PhSe)_2$ (0,5 mg/kg) (v.o.), pela via i.p. salina e não foram expostos à fumaça do cigarro.

Grupo Fumo: os animais receberam óleo de canola (v.o.), pela via i.p. salina e foram expostos à fumaça de 6 cigarros.

Grupo $(PhSe)_2$ + Fumo: os animais receberam por v.o. $(PhSe)_2$ (0,5 mg/kg), pela via i.p. salina e foram expostos à fumaça de 6 cigarros.

Grupo $(PhSe)_2$ + Fumo + BSO: os animais receberam por v.o. $(PhSe)_2$ (0,5 mg/kg), pela via i.p. BSO (1mmol/kg) e foram expostos à fumaça de 6 cigarros.

Para a exposição à fumaça do cigarro, os animais foram colocados em uma câmara de exposição onde foram expostos passivamente à fumaça do cigarro. Essa câmara possui ventilação para a entrada de oxigênio. No centro da câmara foi colocado um aparato no qual foram fixados e queimados os 6 cigarros. Os animais foram expostos à fumaça do cigarro por 15 minutos. Vinte quatro horas após os tratamentos, os animais foram sacrificados e os pulmões e fígado foram retirados para a determinação dos níveis de tióis não-proteicos (SHNP). Todos os tratamentos foram realizados concomitantemente.

8.1.2.2. Níveis de SHNP:

Os níveis de SHNP foram determinados de acordo com o método descrito por Elmam (1959). Para determinar os níveis de NPSH nos pulmões e fígado dos ratos jovens, os tecidos foram homogeneizados em 50 mM de Tris-HCl (1:5 e 1:10, respectivamente). Os homogenatos foram centrifugados a 4000 x g por 10 minutos e os sobrenadantes foram misturados (1:1) com ácido tricloroacético 10 %. Após a centrifugação, o precipitado protéico foi descartado e os níveis de SHNP foram determinados no sobrenadante. Uma alíquota do sobrenadante foi adicionada em um tampão de fosfato de potássio 1M pH 7,4 e 5,5'-ditio-bis(ácido 2-nitrobenzóico) (DTNB) 10 mM. A reação de cor foi determinada em 412 nm.

8.1.3. Resultados

Os resultados demonstram que o $(PhSe)_2$ protegeu a redução dos níveis de SHNP causada pela exposição passiva à fumaça do cigarro no pulmão (Figura 1) e fígado (Figura 2) de ratos jovens. A administração de BSO aboliu o efeito protetor do $(PhSe)_2$ na redução dos níveis de SHNP provocada pela exposição passiva à fumaça do cigarro nos pulmões (Figura 1) e no fígado (Figura 2) de ratos jovens. Além disso, o $(PhSe)_2$ aumentou *per se* os níveis de SHNP no fígado dos animais tratados com o composto.

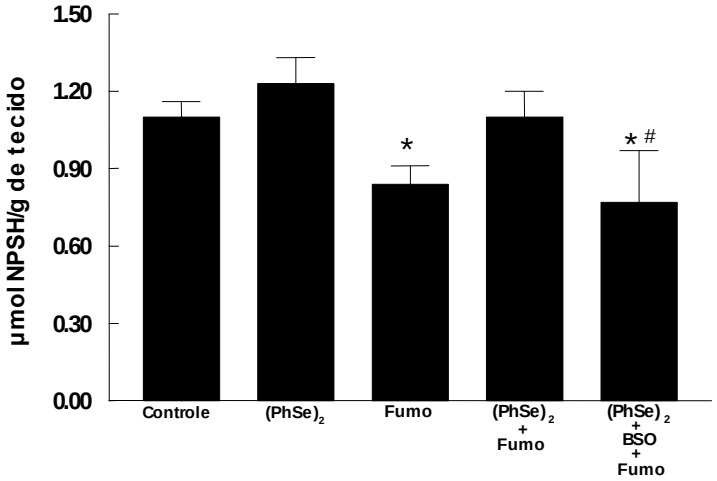


Figura 1: Níveis de SHNP no pulmão. (*) $p < 0.05$ quando comparado com o grupo controle (análise de uma via ANOVA/Duncan). (#) $p < 0.05$ quando comparado com o grupo $(PhSe)_2$ + fumo (análise de uma via ANOVA/Duncan).

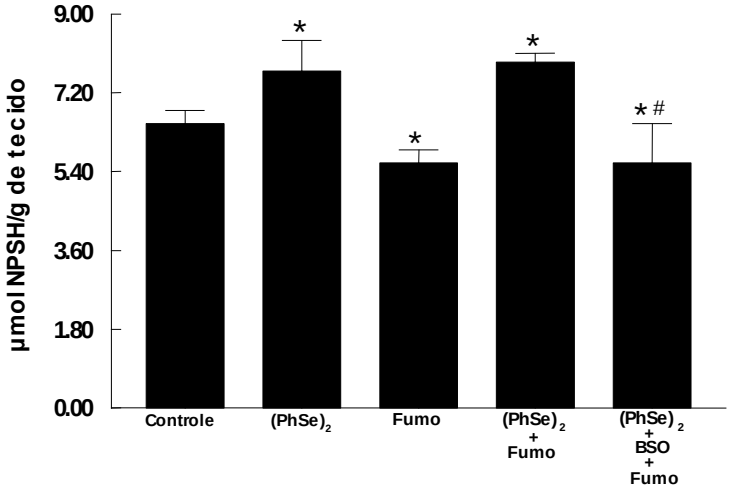


Figura 2: Níveis de SHNP no fígado. (*) $p < 0.05$ quando comparado com o grupo controle (análise de uma via ANOVA/Duncan). (#) $p < 0.05$ quando comparado com o grupo $(PhSe)_2$ + fumo (análise de uma via ANOVA/Duncan).

8.1.4. Conclusão:

Os resultados reforçam a hipótese de que a glutationa, o principal tiol não protéico das células é fundamental para o efeito antioxidante do $(PhSe)_2$.

8.1.5. Referências:

Ellman GL. Tissue sulfhydryl groups. Arch Biochem 1959; 82:70-77.