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

# EFEITOS DE COMPOSTOS ORGÂNICOS DE SELÊNIO FRENTE À TOXICIDADE INDUZIDA POR GLUTAMATO E METILMERCÚRIO

**TESE DE DOUTORADO**

**Cristiane Lenz Dalla Corte**

**Santa Maria, RS, Brasil**
**2012**

# EFEITOS DE COMPOSTOS ORGÂNICOS DE SELÊNIO FRENTE À TOXICIDADE INDUZIDA POR GLUTAMATO E METILMERCÚRIO

**Cristiane Lenz Dalla Corte**

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

**Orientador: Prof. Dr. João Batista Teixeira da Rocha**

**Santa Maria, RS, Brasil**
**2012**

Ficha catalográfica elaborada através do Programa de Geração Automática da Biblioteca Central da UFSM, com os dados fornecidos pelo(a) autor(a).

Dalla Corte, Cristiane Lenz
EFEITOS DE COMPOSTOS ORGÂNICOS DE SELÊNIO FRENTE À TOXICIDADE INDUZIDA POR GLUTAMATO E METILMERCÚRIO / Cristiane Lenz Dalla Corte.-2012.
126 p.; 30cm

Orientador: João Batista Teixeira da Rocha
Tese (doutorado) - Universidade Federal de Santa Maria, Centro de Ciências Naturais e Exatas, Programa de Pós-Graduação em Bioquímica Toxicológica, RS, 2012

1. Compostos orgânicos de selênio 2. Metilmercúrio 3. Disfunção mitocondrial 4. Glutamato 5. Estresse oxidativo I. da Rocha, João Batista Teixeira II. Título.

**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

# EFEITOS DE COMPOSTOS ORGÂNICOS DE SELÊNIO FRENTE À TOXICIDADE INDUZIDA POR GLUTAMATO E METILMERCÚRIO

elaborada por
**Cristiane Lenz Dalla Corte**

**COMISSÃO EXAMINADORA:**

**João Batista Teixeira da Rocha, Dr.**
(Presidente/ Orientador)

**Jeferson Luis Franco, Dr.** (UNIPAMPA)

**Robson Luiz Puntel, Dr.** (UNIPAMPA)

**Marta Maria Medeiros Frescura Duarte, Dra.** (ULBRA)

Ricardo Brandão
**Ricardo Brandão, Dr.** (UFSM)

Santa Maria, 2 de março de 2011.

## AGRADECIMENTOS

Agradeço aos meus pais José Erom e Lourença e ao meu irmão Jefferson pelo incentivo, pela sua dedicação, pelos seus ensinamentos e pelo apoio em todos os momentos.

Ao meu orientador, Prof. João Batista Teixeira da Rocha pela orientação, pela paciência e principalmente pelos ensinamentos ao longo da minha formação acadêmica.

Agradeço ao Félix pelo amor, carinho, amizade e companheirismo em todos os momentos, e a sua família pela amizade e apoio em todos os momentos.

Aos amigos e colegas do Laboratório do Prof. João. Obrigada pelo carinho, companheirismo e pelo conhecimento compartilhado.

Aos amigos e colegas do Laboratório do Prof. Félix. Obrigada pela amizade e companheirismo.

Aos amigos e colegas do Laboratório da Profa. Nilda. Obrigada pela amizade e companheirismo.

Em especial gostaria de agradecer aqueles que contribuíram para a realização deste trabalho: Carol, Jéssie, Fernando, Luíza, Gerlânia, Bruna, Roselei e Alcindo. Sem vocês a realização deste trabalho não seria possível.

Aos demais professores do Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica.

Aos funcionários do Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica.

Ao CNPq e demais agências de fomento pela bolsa de estudos e pelo suporte financeiro.

Aos animais utilizados, todo o meu respeito, pois, sem eles a realização deste trabalho não seria possível.

Enfim agradeço à Universidade Federal de Santa Maria e ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica pela possibilidade de realização deste curso.

## RESUMO

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

# EFEITOS DE COMPOSTOS ORGÂNICOS DE SELÊNIO FRENTE À TOXICIDADE INDUZIDA POR GLUTAMATO E METILMERCÚRIO

AUTORA: Cristiane Lenz Dalla Corte
ORIENTADOR: João Batista Teixeira da Rocha
LOCAL E DATA DA DEFESA: Santa Maria, Março de 2012.

Os compostos orgânicos de selênio têm sido propostos como potenciais agentes farmacológicos para o tratamento de patologias associadas ao estresse oxidativo. Em particular, o disseleneto de difenila e o ebselen têm sido demonstrados como potentes agentes antioxidantes em uma variedade de modelos experimentais. Dessa forma, os objetivos do presente estudo foram: avaliar os efeitos do ebselen e do disseleneto de difenila, bem como, da sua combinação com a guanosina, sobre o estresse oxidativo induzido por glutamato em diferentes regiões do cérebro de rato (artigo 1); testar os efeitos do disseleneto de difenila frente à disfunção mitocondrial induzida por metilmercúrio (MeHg) em fatias de fígado de rato (manuscrito 1); e investigar os efeitos do MeHg (5 mg/ kg/ dia, i.g.) e/ ou disseleneto de difenila (1 mg/ kg /dia, i.p.) sobre a disfunção mitocondrial, a atividade da tioredoxina redutase (TrxR), os níveis de mercúrio, e a atividade locomotora após 21 dias de tratamento em ratos (manuscrito 2). No artigo 1 foi observado que os compostos orgânicos de selênio (ebselen e disseleneto de difenila) e a guanosina foram capazes de reduzir a produção de espécies reativas de oxigênio (EROS) induzida por glutamato em fatias de córtex, estriado e hipocampo de rato quando usados isolados ou combinados. A guanosina também preveniu a inibição da captação de glutamato nas regiões do cérebro testadas. A combinação dos compostos orgânicos de selênio com a guanosina foi mais efetiva na proteção contra a produção de EROS induzida por glutamato do que cada composto separadamente. Os resultados do manuscrito 1 mostram que o disseleneto de difenila na concentração mais baixa (0,5 µM) preveniu contra a disfunção mitocondrial induzida por MeHg em fatias de fígado de rato, a qual pode estar associada com a interação do intermediário selenol do disseleneto de difenila com o MeHg formando um complexo inerte, bem como com as propriedades antioxidantes do intermediário selenofenol. No manuscrito 2, foi observado que o co-tratamento com disseleneto de difenila protegeu contra a depleção de tióis pelo MeHg em mitocôndrias de cérebro e fígado de rato mas, não preveniu a disfunção mitocondrial cerebral e hepática induzida por MeHg, nem protegeu contra a inibição da atividade da TrxR por MeHg em cérebro, fígado e rim. Além disso, o co-tratamento com disseleneto de difenila causou um maior acúmulo de Hg no cérebro e fígado, e aumentou os déficits motores e a perda de peso corporal. Em conjunto, os resultados apresentados aqui reforçam o papel central da disfunção mitocondrial na toxicidade induzida pelo MeHg tanto *in vitro* quanto *in vivo* e o papel da TrxR como um alvo molecular para o MeHg em ratos. Além disso, os resultados indicam que os compostos orgânicos de selênio, como o ebselen e o disseleneto de difenila, são agentes promissores contra o dano oxidativo induzido tanto por glutamato quanto por MeHg *in vitro*, no entanto deve-se ter muita cautela ao extrapolar estes resultados para situações *in vivo*, uma vez que o co-tratamento com disseleneto de difenila e MeHg aumentou a neurotoxicidade em ratos.

**Palavras-chave:** Compostos orgânicos de selênio. Metilmercúrio. Disfunção mitocondrial. Glutamato. Estresse oxidativo.

## ABSTRACT

Thesis of PhD's Degree
Graduate Course in Toxicological Biochemistry
Federal University of Santa Maria, RS, Brazil

### EFFECTS OF ORGANOSELENIUM COMPOUNDS AGAINST THE TOXICITY INDUCED BY GLUTAMATE OR METHYLMERCURY

AUTHOR: Cristiane Lenz Dalla Corte
ADVISOR: João Batista Teixeira da Rocha
PLACE AND DATE OF THE DEFENSE: Santa Maria, March, 2012.

Organoselenium compounds have been proposed as potential pharmacological agents in the treatment of diseases associated with the oxidative stress. Particularly, ebselen and diphenyl diselenide has been demonstrated as potential antioxidant agents in a variety of experimental models. Thus, the aims of the present study were: to evaluate the effect of ebselen and diphenyl diselenide, as well as, their combination with guanosine on the oxidative stress induced by glutamate in different regions of rat brains (article 1), to test the effects of diphenyl diselenide on mitochondrial dysfunction induced by methylmercury (MeHg) in rat's liver slices (manuscript 1), and investigate the effects of MeHg (5 mg/ kg/ day, i.g.) and/ or diphenyl diselenide (1 mg/ kg /day, i.p.) on mitochondrial dysfunction, thioredoxin reductase (TrxR) activity, mercury levels and locomotor activity after 21 days of treatment in rats (manuscript 2). In article 1, the organoselenium compounds (ebselen and diphenyl diselenide) and guanosine were able to reduce the glutamate-induced reactive oxygen species (ROS) production in cortex, striatum and hippocampus of rat when used isolated or in combination. Guanosine also prevented glutamate uptake in the regions of rat brains tested. The combination of guanosine with organoselenium compounds was more effective against glutamate-induced ROS production than the individual compounds alone. The results in manuscript 1, show that diphenyl diselenide, at low concentrations (0.5 µM), protected against the MeHg-induced mitochondrial dysfunction in liver slices, which may be associated with the interaction between diphenyl diselenide's selenol intermediate and MeHg, with the formation of inert complex(es), as well as, with the antioxidant properties of the selenophenol intermediate. In manuscript 2, it was observed that diphenyl deiselenide protected against thiol depletion by MeHg in cerebral and hepatic mitochondria, but it didn't prevent the mitochondrial dysfunction induced by MeHg in brain and liver, nor protected from TrxR activity inhibition by MeHg in brain, liver and kidney. Moreover, the co-treatment with MeHg and diphenyl diselenide caused an increase in mercury deposition in brain and liver, increased the motor deficits and the loss of body weight. Taken together, the results present here reinforce the central role of the mitochondrial dysfunction on the toxicity induced by MeHg both *in vivo* and *in vitro*, and the role of TrxR as a molecular target for MeHg in rats. Moreover, the results indicate that organoselenium compounds, such as ebselen and diphenyl diselenide, are promising agents against the oxidative damage induced by both glutamate and MeHg *in vitro*, however, caution must be taken on the extrapolation of these results to *in vivo* situations, since the co-treatment with diphenyl diselenide and MeHg increased the neurotoxicity in rats.


**Keywords:** Organoselenium compounds. Methylmercury. Mitochondrial dysfunction. Glutamate. Oxidative stress.

# LISTA DE FIGURAS

**INTRODUÇÃO**



**ARTIGO 1**



**MANUSCRITO 1**

**MANUSCRITO 2**

# LISTA DE TABELAS

# LISTA DE ABREVIATURAS

ΔΨm – potencial de membrada mitocondrial

δ-ALA-D – δ-aminolevulinato desidratase

AMPA – ácido α-amino-3-hidroxi-5-metil-4-isoxazol-propiônico

ATP – adenosina trifosfato

AVC – acidente vascular cerebral

DTT – DL-ditiotreitol

EAATs – transportadores de aminoácidos excitatórios

EROs – espécies reativas de oxigênio

GPx – glutationa peroxidase

GSH – glutationa reduzida

GluR – receptores glutamatérgicos

$HgCl_2$ – cloreto de mercúrio

$H_2O_2$ – peróxido de hidrogênio

iGluRs – receptores ionotrópicos

KA – cainato

MeHg – metilmercúrio

mGluRs – receptores metabotrópicos

MTT – brometo de 3-[4,5-dimetil-tiazol-2-il]-2,5-difenil- tetrazólio

NADPH – Nicotinamida adenina dinucleotideo fosfato reduzido

NMDA – *N*-metil-*D*-aspartato

NO – óxido nítrico

NOS – óxido nítrico sintase

PLC – fosfolipase C

PTPM – poro de transição de permeabilidade mitocondrial

SeH – selenoidrila

SH – sulfidrila

SNC – sistema nervoso central

Trx – tioredoxina

TrxR – tioredoxina redutase

**SUMÁRIO**

## APRESENTAÇÃO

No item **INTRODUÇÃO**, está descrita uma sucinta revisão bibliográfica sobre os temas trabalhados nesta tese.

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

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

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

# 1. INTRODUÇÃO

## 1.1. Selênio

O elemento selênio, juntamente com o oxigênio, o enxofre, o telúrio, e o polônio, faz parte do grupo 16 (antigamente grupo 6A), ou grupo dos Calcogênios da tabela periódica. Consequentemente, o selênio compartilha com o enxofre e o telúrio algumas propriedades físicas e químicas. Inicialmente, o selênio foi reconhecido como um elemento tóxico. O primeiro caso bem documentado de intoxicação por selênio em mamíferos foi em decorrência da ingestão de pastagens com altos níveis de selênio por cavalos no Fort Randall nos EUA em 1856. Além disso, a toxicidade do selênio foi demonstrada experimentalmente no inicio do século XX (Franke 1934a, b; Franke e Painter, 1935; Painter, 1941; Moxon e Rhian, 1943), e foi associada com os casos de intoxicação relatados anteriormente.

Apenas no início dos anos 1950, um estudo pioneiro de Schwarz e Foltz, forneceu as observações iniciais de que o selênio pode prevenir a necrose hepática em ratos alimentados com uma dieta deficiente de selênio (Schwarz e Foltz 1957). A associação entre o selênio e patologias do fígado levou rapidamente ao reconhecimento de que o selênio é um elemento traço nutricionalmente importante (Oldfield, 1987; Navarro-Alarcon e Cabrera-Vique, 2008). A identificação do aminoácido selenocisteína no centro ativo da enzima glutationa peroxidase (GPx) hepática de ratos trouxe o reconhecimento de um novo nucleófilo, ex: o selenolato. Este grupo participa como um poderoso agente redutor em enzimas antioxidantes, tais como a GPx e a tioredoxina redutase (TrxR) (Flohe e cols., 1973; Rotruck e cols., 1973; Oh e cols., 1974; Flohe, 1988; Allan e cols., 1999; Lu e Holmgren, 2009; Nogueira e Rocha 2010).

### 1.1.1. Compostos orgânicos de selênio

Desde a descoberta de que o selênio poderia desempenhar um papel fundamental na atividade de enzimas antioxidantes o interesse por compostos orgânicos contendo selênio cresceu bastante, principalmente pelo fato de que estes poderiam imitar a química fisiológica redox dos grupos selenol/ selenolato, podendo complementar as defesas naturais das células contra agentes oxidantes (Rotruck e cols., 1973; Nogueira e cols., 2004). Dessa forma, compostos orgânicos de selênio síntéticos poderiam representar uma nova estratégia terapêutica para doenças relacionadas com o estresse oxidativo (Parnham e Graf, 1991; Arteel e Sies, 2001; Mugesh e cols., 2001; Nogueira e cols., 2004). O estresse oxidativo caracteriza-se por um desequilibrio entre a produção de espécies reativas de oxigênio (EROs) e a sua remoção por sistemas antioxidantes (Ali e cols., 1992; Yee e Choi, 1996; Sarafian, 1999;

Sanfeliu e cols., 2001). A superprodução de EROs pode danificar macromoléculas biológicas, tais como ácidos nucléicos, proteínas, lipídios e carboidratos (Xiong e cols., 2007).

#### 1.1.1.1. Ebselen

O primeiro composto orgânico de selênio relatado na literatura com atividade mimética da GPx foi o ebselen (2-fenil-1,2-benzisoselenazol-3[2H]-ona) (Figura 1). O ebselen na presença de glutationa reduzida (GSH) catalisa a redução de uma ampla variedade de hidroperóxidos e pode ajudar o sistema de defesa celular contra o estresse oxidativo (Muller e cols., 1984; Parnham e Kindt, 1984; Wendel e cols., 1984). O mecanismo da redução catalítica de hidroperóxidos desempenhado pelo ebselen foi proposto por Maiorino e colaboradores e pareceu cineticamente idêntico ao da reação enzimática da GPx (Maiorino e cols., 1988). O ebselen reage com os tióis para produzir um selenenil sulfeto (Figura 2). O selenenil sulfeto reage com um segundo equivalente de GSH para produzir um único produto que é caracterizado como selenol. Finalmente, o selenol reage com peróxido de hidrogênio ($H_2O_2$) ou hidroperóxido orgânico para formar $H_2O$, ou o respectivo álcool (ROH), e o ácido selenênico do ebselen, o qual produz uma outra molécula de $H_2O$ e regenera o ebselen (Nogueira e Rocha, 2011).

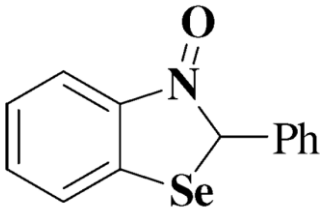



Figura 1. Estrutura do ebselen.

Além da atividade antioxidante, o ebselen demonstrou possuir propriedades antiinflamatória, antinociceptiva, neuroprotetora e anti-úlcera em vários modelos animais (Maiorino e cols., 1992; Nogueira e cols., 2004). Na verdade, a demonstração clínica de que o ebselen apresentou efeitos neuroprotetores contra as condições patológicas associadas com a isquemia cerebral cerca de uma década atrás (Saito e cols., 1998; Yamaguchi e cols., 1998; Ogawa e cols., 1999; Parnham e Sies, 2000) e os extensos dados obtidos em modelos animais demonstrando que o ebselen foi efetivo contra os efeitos deletérios causados por isquemia/ reperfusão reforçam ainda mais a importância do estudo da farmacologia e toxicologia dos compostos orgânicos de selênio (Dawson e cols., 1995; Ozaki e cols., 1997; Takasago e cols.,

1997; Imai e cols., 2001; Lapchak e Zivin, 2003; Hamacher e cols., 2009; Seo e cols., 2009; Tunc e cols., 2009; Gul e cols., 2010).

Evidências indicam que as propriedades antioxidante e farmacológica do ebselen são, em grande parte, devido às suas reações com o sistema da tioredoxina (Trx) (Zhao e Holmgren, 2002). O ebselen pode ser reduzido tanto pela tioredoxina redutase (TrxR) na presença de NADPH quanto pela Trx reduzida para formar um selenol, que reage com hidroperóxidos produzindo um derivado ácido selenênico. O selenol também pode sofrer uma rápida oxidação para formar um disseleneto que atua como um substrato para a TrxR de mamíferos, formando como produto final um selenol ativo (Zhao e Holmgren, 2002; Zhao e cols., 2002).

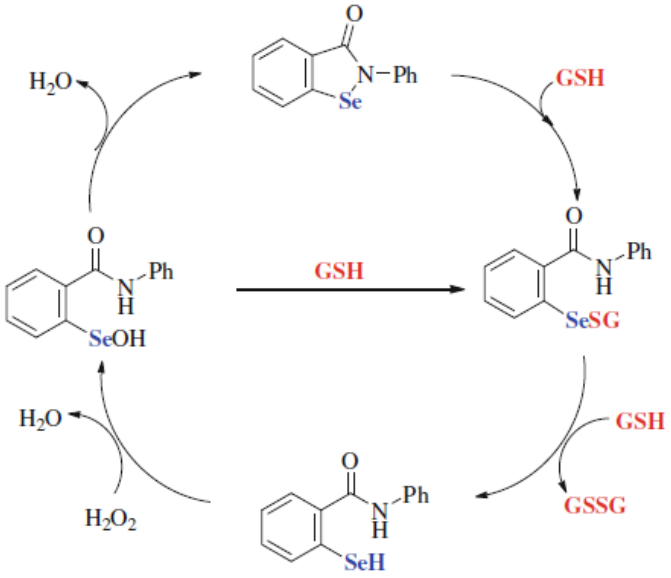



Figura 2. Mecanismo de redução catalítica de hidroperóxidos pelo ebselen. Fonte: Nogueira e cols., 2004.

#### 1.1.1.2. Disseleneto de difenila

O disseleneto de difenila (Figura 3), o mais simples diaril disseleneto, é bastante empregado como intermediário na síntese orgânica. A possibilidade de uma possível contamição ocupacional motivou estudos toxicológicos com este composto. No entanto, a descoberta de que o disseleneto de difenila possui uma maior atividade tiol-peroxidase que o ebselen (Wilson e cols., 1989) além de apresentar uma menor toxicidade em roedores (Meotti e cols., 2003; Nogueira e cols., 2003), motivou estudos a respeito da farmacologia deste composto.

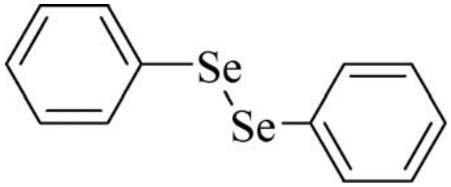



Figura 3. Estrutura do disseleneto de difenila.

Nogueira e cols. (2003) demonstrou que o disseleneto de difenila possui atividade antinociceptiva e antiiflamatória melhores que o ebselen. Outras atividades biológicas do disseleneto de difenila também foram relatadas durante a última década como hepatoprotetora (Borges et al., 2005, 2006), nefroprotetora (Brandão et al., 2009), anti-úlcera (Savegnago et al., 2006, Ineu et al., 2008), cardioprotetora (de Bem et al., 2009; Rocha et al., 2009), neuroprotetora (Rosa et al., 2003; Stangherlin et al., 2008; Burger et al., 2006), anti-carcinogênica (Barbosa et al., 2008), anti-hiperglicêmica (Barbosa et al., 2006), anxiolítica (Savegnago et al., 2008) e antidepressiva (Ghisleni et al., 2008; Savegnago et al., 2008). Dessa forma, este composto tem emergido como um candidato para diferentes fins terapêuticos.

Outra importante aplicação do disseleneto de difenila seria na redução da deposição de mercúrio (Nogueira e Rocha, 2010). O grupo selenol possui uma alta afinidade pelo mercúrio podendo interagir com determinados compostos mercuriais (Sugiura e cols., 1976). Consequentemente, a toxicidade dos compostos mercuriais pode ser modificada pela sua interação com grupos selenóis e tendo em vista o fato de que o disseleneto de difenila pode ser reduzido no seu intermediário selenol após a interação com tióis, este composto poderia alterar a toxicidade dos compostos mercuriais após exposição *in vivo* (Nogueira and Rocha, 2010, 2011; Farina e col., 2011a).

Recentemente foi demonstrado que a exposição simultânea ao disseleneto de difenila e ao MeHg causou uma redução acentuada na deposição de mercúrio no fígado, rim e cérebro de camundongos (de Freitas e cols., 2009). Foi proposto que o intermediário selenol formado após a redução do disseleneto de difenila poderia reagir com MeHg para formar um complexo mais excretável do tipo $CH_3HgSePh$, o que reduziu consideravelmente a deposição de mercúrio em camundongos adultos (de Freitas e cols., 2009). Além disso, as propriedades antioxidantes do disseleneto de difenila também podem contribuir para a proteção contra o estresse oxidativo induzido por MeHg, como demonstrado em frações de cérebro de camundongo ricas em mitocôndrias (Meinerz e col., 2011). No entanto, outro trabalho

demonstrou que a exposição aguda e concomitante ao cloreto de mercúrio ($HgCl_2$) e disseleneto de difenila causou 100% de mortalidade e potencializou o dano renal em camundongos, sugerindo um efeito sinérgico tóxico entre $Hg^{2+}$ e selênio relacionado com um complexo formado entre $Hg^{2+}$ e disseleneto de difenila o qual poderia ter atividade pró-oxidante (Brandão e cols., 2006, 2010). Por outro lado, pré- e pós-tratamentos subcrônicos com disseleneto de difenila foram eficazes na proteção contra as alterações imunológicas e hematológicas induzidas por $HgCl_2$ em camundongos (Brandão e cols., 2008).

O mecanismo catalítico proposto para a ação antioxidante do disseleneto de difenila envolve a interação direta com tióis de baixo peso molecular gerando grupos selenóis intermediários (Figura 4) (Nogueira e cols., 2004). De um modo semelhante ao que ocorre no centro ativo da GPx, o grupo selenol/ selenolato formado pode decompor o $H_2O_2$ e peróxidos lipídicos (Nogueira e Rocha, 2010). Consequentemente, as propriedades farmacológicas do disseleneto de difenila podem estar associadas com as suas atividades antioxidantes que dependem grandemente da formação de um grupo selenol após a interação com tióis reduzidos (Nogueira e cols., 2004). Recentemente, foi demonstrado que o disseleneto de difenila, da mesma forma que o ebselen, poderia ser substrato da TrxR cerebral e hepática de rato (de Freitas e Rocha, 2010, 2011). Portanto as propriedades antioxidantes do disseleneto de difenila poderiam, pelo menos em parte, estar associadas a sua redução em selenofenol pela TrxR.

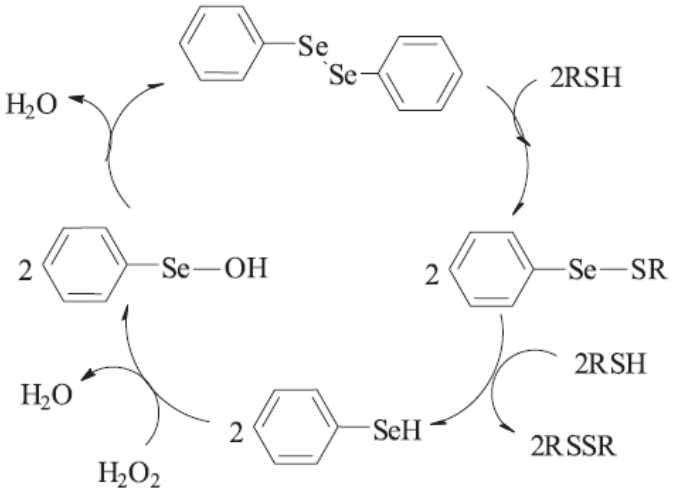



Figura 4. Mecanismo catalítico para a ação antioxidante do disseleneto de difenila. Fonte: Nogueira e Rocha, 2010.

### 1.1.1.3. Toxicologia dos compostos orgânicos de selênio

Embora a atividade do tipo tiol-peroxidase dos compostos orgânicos de selênio seja importante para suas propriedades antioxidantes, também pode contribuir para suas propriedades toxicológicas devido à oxidação de tiois a partir de GSH ou proteínas contendo tiol. No caso de enzimas isto pode resultar na perda da atividade catalítica (Nogueira e cols., 2004). A interação de diferentes disselenetos de arila com tióis de baixo peso molecular e com a enzima sulfidrílica δ-aminolevulinato desidratase (δ-ALA-D) de mamíferos foram demonstrados por diferentes trabalhos (Barbosa e cols., 1998; Maciel e cols., 2000; Nogueira e cols., 2004; Folmer e cols., 2005).

De significando toxicológico importante, tanto o ebselen quanto o disseleneto de difenila podem induzir disfunção mitocondrial *in vitro*, o que também foi associado com a oxidação de importantes grupos tióis na mitocôndria (Morin e cols., 2003; Puntel e cols., 2010; Yang e cols., 2000a, b).

Além disso, têm sido demonstrado que o disseleneto de difenila pode causar efeitos neurotóxicos como convulsões em roedores, dependendo da dose administrada, do veículo, da via de administração e da idade dos animais (Nogueira e cols., 2001, 2002; Moretto e cols., 2003, 2007; Prigol e cols., 2009; Nogueira e Rocha, 2011).

### 1.2. Glutamato

O aminoácido L-glutamato é o principal neurotransmissor excitatório do sistema nervoso central (SNC) de mamíferos exercendo um papel fundamental na maioria das funções do sistema nervoso incluindo a cognição, a memória e o aprendizado, e também no desenvolvimento do SNC (Fonnum, 1984; Collingridge e Lester, 1989;Headley e Grillner, 1990; Danbolt, 2001). Quase todos os neurônios excitatórios no SNC são glutamatérgicos e estima-se que mais da metade de todas as terminações nervosas liberem este aminoácido excitatório (Walton e Dodd, 2007).

O glutamato pode ser sintetizado, nos terminais nervosos, tanto a partir da glicose via ciclo de Krebs e transaminação do α-cetoglutarato, quanto a partir da glutamina, produzida nas células gliais, que é levada aos neurônios e convertida pela glutaminase em glutamato (Figura 5) (Deutch e Roth, 1999; Danbolt, 2001). Nos terminais nervosos, o glutamato é armazenado dentro de vesículas sinápticas por um processo dependente de $Mg^{2+}$. Quando os terminais glutamatérgicos pré-sinapticos são despolarizados, o glutamato vesicular é liberado por exocitose na fenda sináptica de forma dependente de $Ca^{2+}$ (Greene e Greenamyre, 1996). Tanto a liberação de glutamato quanto o seu armazenamento nas vesículas sinápticas são processos dependentes de ATP, ou seja, acarretam dispêndio de energia (Danbolt, 2001). A

liberação de glutamato causa uma mudança de 1000 vezes na concentração de glutamato na fenda sináptica (Nicholls e Attwell, 1990). O glutamato então, se liga aos receptores pós-sinápticos ionotrópicos, estimulando o influxo de cátions para despolarizar a célula pós-sináptica (Walton e Dodd, 2007).

Após a sua liberação o glutamato deve ser removido da fenda sináptica de forma rápida e eficiente de modo a evitar o excesso de estimulação dos seus receptores. O glutamato extracelular é então captado por transportadores dependentes de $Na^+$ de alta afinidade localizados em neurônios e astrócitos (Figura 5) (Maragakis e Rothstein, 2004). O glutamato captado pelos astrócitos é então convertido em glutamina pela glutamina sintetase, podendo ser transferida para os neurônios para ser convertida em glutamato pela glutaminase ativada por fosfato (Danbolt, 2001).

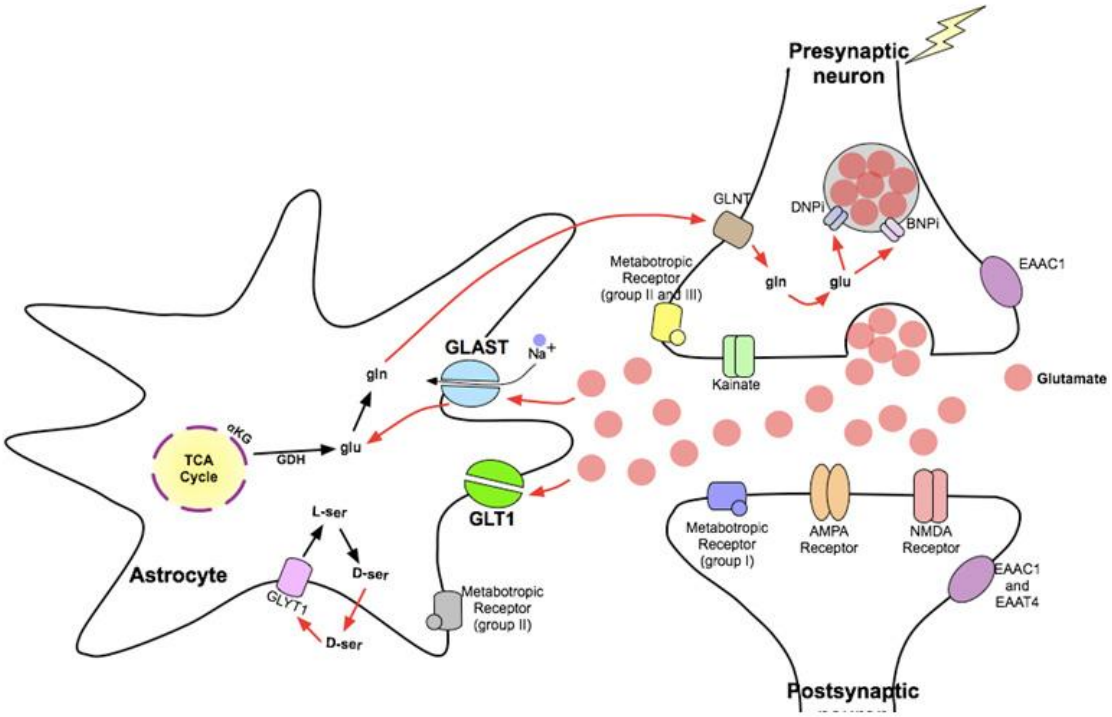



Figura 5. Sinapse glutamatérgica. Fonte: http://people.usd.edu/~cliff/Courses/Behavioral%20Neuroscience/Transmitters/transmitterfigs/transmitters.html

### 1.2.1. Receptores glutamatérgicos

Os receptores de glutamato são encontrados em ambas as membranas celulares neuronal e glial. Os receptores glutamatérgicos ionotrópicos mediam diretamente o influxo de cátions na célula pós-sináptica, levando a despolarização da membrana (Figura 6). Os

receptores glutamatérgicos metabotrópicos atuam indiretamente por meio de segundos mensageiros intracelulares (Figura 6) (Walton e Dodd, 2007).

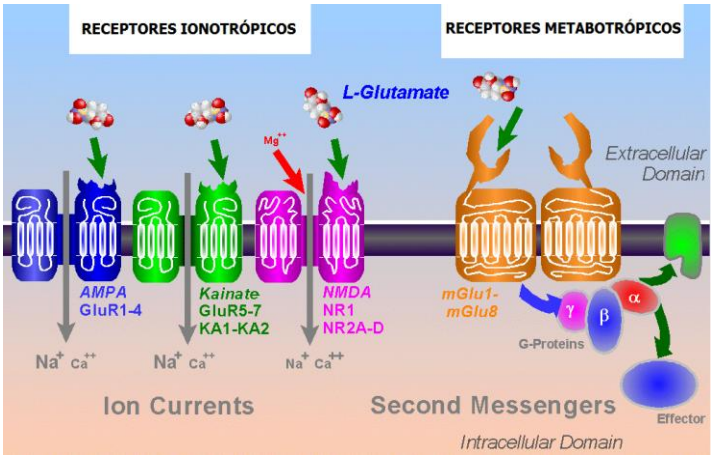



Figura 6. Receptores glutamatérgicos. Fonte: http://www.ucl.ac.uk/~smgxt01/frameh.htm?page=glutamat.htm

#### 1.2.1.1. Receptores glutamatérgicos ionotrópicos

A neurotransmissão glutamatérgica é alcançada rapidamente através da ativação das três classes de receptores ionotrópicos (iGluRs) que são nomeados de acordo com suas seletividades a agonistas específicos: *N*-metil-*D*-aspartato (NMDA), cainato (KA) e ácido α-amino-3-hidroxi-5-metil-4-isoxazol-propiônico (AMPA) (Nakanishi, 1992; Ozawa e cols., 1998; Simeone e cols., 2004). Os receptores ionotrópicos podem ainda ser divididos em receptores NMDA e não-NMDA. Os receptores não-NMDA, são ativados por AMPA e KA, formam canais permeáveis principalmente aos íons $Na^+$ e $K^+$ e são responsáveis pela neurotransmissão sináptica excitatória rápida (Tang e cols., 1989; Trussell e Fischbach, 1989; Sommer e Seeburg, 1992; Heath e Shaw, 2002). Os receptores ativados por NMDA são permeáveis preferencialmente ao $Ca^{2+}$, possuindo uma cinética mais lenta com a abertura dos canais persistindo por várias dezenas a centenas de milisegundos (Hudspith, 1997). Os receptores glutamatérgico têm diferentes distribuições corticais, perfis farmacológicos, e funções na neurotransmissão glutamatérgica.

Os receptores NMDA possuem cinco diferentes sítios de ligação para ligantes endógenos que influenciam a abertura dos canais iônicos (Ozawa e cols., 1998). Dois destes sítios de ligação são para os co-agonistas glutamato e glicina (Kleckner e Dingledine, 1988). A ligação desses co-agonistas é necessária para a abertura total do canal iônico. A ligação do

$Mg^{2+}$ a um sítio específico no receptor bloqueia o canal iônico (Nowak e cols., 1984). Este bloqueio pode ser superado de forma dependente de voltagem, a partir de despolarizações ocasionadas pela ativação prévia dos receptores AMPA e KA (Walton e Dodd, 2007). Existem ainda um sítio regulatório para poliamidas, e um para o zinco que age inibindo o fluxo iônico, além de um sítio redox na face externa do receptor onde grupos sulfidrílicos (-SH) podem interagir com derivados do óxido nítrico (NO) alterando a função do receptor (Aizenman e cols., 1989; Lei e cols., 1992; Rock e Macdonald, 1992). A oxidação leva a redução da entrada de $Ca^{2+}$ através dos canais de NMDA e tem sido proposto como um mecanismo protetor contra a superestimulação do receptor NMDA sob condições de estresse oxidativo (Aizenman e cols., 1990; Trotti e cols., 1998).

Os receptores AMPA existem em complexos tetraméricos compostos de subunidades GluR 1 a 4 (Palmer e cols., 2005). Os receptores AMPA em sua maioria permitem o influxo de $Na^{+}$ e $K^{+}$ após a ligação do glutamato. A subunidade GluR2 dos receptores AMPA, em sua forma editada, é de particular interesse uma vez que a sua presença no complexo do receptor AMPA resulta em uma permeabilidade ao $Ca^{2+}$ extremamente baixa (Hollmann e cols., 1991; Brorson e cols., 1999). Os receptores AMPA que não contêm uma subunidade GluR2 se assemelham a receptores NMDA na medida em que permitem um fluxo significativo de íons $Ca^{2+}$. Embora sejam poucos em número, os receptores AMPA permeáveis a $Ca^{2+}$ poderiam estar envolvidos nos mecanismos excitotóxicos (Kwak e Weiss, 2006).

Os receptores KA são compostos de duas subfamílias GluR5-7 e KA-1 e -2, e estão envolvidos em ambos os tipos de neurotransmissão, excitatória e inibitória, nas células piramidais do hipocampo (Mulle e cols., 2000; Walton e Dodd, 2007). A ligação do KA aos seus receptores ativa canais de $Na^{2+}$ com cinética similar aos receptores AMPA (Huettner, 1990, 2003). O KA também pode se ligar com menor afinidade a receptores AMPA causando a sua ativação (Hudspith, 1997).

#### 1.2.1.2. Receptores glutamatérgicos metabotrópicos

Os receptores metabotrópicos pertencem a familia de receptores acoplados a proteína G, e medeiam uma variedade de funções, incluindo a excitabilidade neuronal, a transmissão sináptica e a plasticidade neuronal, dependendo de sua associação com segundos mensageiros (Walton e Dodd, 2007). Atualmente, oito subtipos de receptores metabotrópicos (mGluRs) já foram descritos, podendo ser agrupados em três classes distintas com base nos mecanismos de transdução de sinal intracelular e na afinidade por agonistas farmacológicos: grupo I (mGluR1 e 5), grupo II (mGluR2 e 3) e grupo III (mGluR4 e 6-8) (Okamoto e cols., 1994; Duvoisin e

cols., 1995). Os receptores mGluRs do grupo I podem provocar a liberação de $Ca^{2+}$ intracelular através da ativação da fosfolipase C (PLC) que produz inositol-1,4,5-trifosfato e diacilglicerol como segundos mensageiros (Bruno e cols., 2001). Já os receptores dos grupos II e III são acoplados a adenilato ciclase (Meldrum, 2000). Os receptores mGluRs estão localizados nos terminais pré- e pós-sinápticos e nas células gliais e sua ativação pode promover efeitos excitatórios e inibitórios (Ozawa e cols., 1998).

### 1.2.2. Transportadores de glutamato

Após a sua liberação na fenda sináptica, o glutamato não é metabolizado por enzimas extracelulares, mas sim removido através da captação celular (Trotti e cols., 1998). Até o presente momento cinco isoformas de "transportadores de glutamato acoplados a $Na^+$ e $K^+$" ou "transportadores de aminoácidos excitatórios" (EAATs) foram identificados e nomeados: GLAST (EAAT1), GLT1 (EAAT2), EAAC1 (EAAT3), EAAT4 e EAAT5 (Kanai e Hediger, 1992; Pines e cols., 1992; Storck e cols., 1992; Fairman e cols., 1995; Arriza e cols., 1997). Os EAATs são encontrados na membrana plasmática de vários tipos de células do SNC e são utilizados para remover o glutamato do espaço extracelular (Kanai e Hedinger, 2003; Maragakis e Rothstein, 2004).

A atividade dos transportadores de glutamato depende do gradiente eletroquímico gerado pela $Na^+/K^+$-ATPase na mesma membrana (Szatkowski e cols., 1990; Attwell e cols., 1993). Uma molécula de glutamato é transportada para o citoplasma junto com 2-3 íons $Na^+$ e um $H^+$, enquanto um íon $K^+$ é transportado para o meio extracelular (Zerangue e Kavanaugh, 1996; Kanai e Hediger, 2003). Esta estequiometria lhes permite promover uma alta concentração de glutamato no interior das células e favorece a conservação de energia (Zerangue e Kavanaugh, 1996; Attwell e Laughlin, 2001). A restauração do gradiente eletroquímico de $Na^+$ após a captação de aminoácidos ocorre com o gasto de ATP consumido pela $Na^+/K^+$-ATPase na membrana (Bonvento e cols., 2002). Portanto a manutenção dos níveis energéticos é essencial para o controle da transmissão glutamatérgica (Wadiche e cols., 1995; Billups e cols., 1996). Além de serem co-transportadores de glutamato, estas cinco proteínas também funcionam como canais de $Cl^-$. O influxo de $Cl^-$ é ativado pelo glutamato, mas não é termodinamicamente acoplado ao transporte (Fairman e cols., 1995).

A captação de glutamato é realizada principalmente pelos transportadores GLT-1 e GLAST (Anderson e Swanson, 2000; Danbolt, 2001). Em um cérebro desenvolvido normal, os transportadores GLT-1 e GLAST são encontrados apenas nas membranas dos astrócitos intimamente associados com contatos sinápticos excitatórios, e são responsáveis por manter

baixas as concentrações de glutamato extracelular (Chaudhry e cols., 1995). Em particular, o transportador GLT-1, é responsável por 60% de toda a atividade de captação do glutamato extracelular (Haugeto e cols., 1996; Rothstein e cols., 1996; Tanaka e cols., 1997; Robinson, 1998; Danbolt, 2001).

Notou-se em meados dos anos 1980 que a captação de glutamato é sensível a oxidantes de grupos-SH, incluindo o mercúrio, e que a inibição deste é independente da $Na^+/K^+$-ATPase (Ogita e Yoneda, 1986; Brookes, 1988; Aschner e cols., 1990) e é, em parte reversível por DL-ditiotreitol (DTT) (Albrecht e cols., 1993). Dados sugerem que os transportadores de glutamato possuem um mecanismo regulatório redox baseado no -SH (Trotti e cols., 1997b). A interconversão redox de grupos -SH nos transportadores reduz a Vmax do transporte de glutamato sem afetar o Km e sem afetar a condutância do $Cl^-$ (Trotti e cols., 1997a). Isto implica que a translocação de glutamato e a de vias de permeação iônica da molécula do transportador são separados e submetidos a modulação independente.

### 1.2.3. Excitotoxicidade

A superestimulação dos receptores glutamatérgicos devido ao acúmulo de glutamato na fenda sináptica pode levar a um processo conhecido como excitotoxicidade glutamatérgica que resulta em morte neuronal (Lucas e Newhouse, 1957; Olney, 1969; Coyle e cols., 1981; Choi, 1992). A excitotoxicidade do glutamato tem sido implicada em uma série de distúrbios neurológicos, incluindo acidente vascular cerebral (AVC), epilepsia, lesão cerebral traumática, doenças neurodegenerativas e exposição a toxinas ambientais (Meldrum, 1990; Meldrum e Garthwaite, 1990; Bradford, 1995; Zauner e Bullock, 1995).

A ativação excessiva e prolongada dos receptores de glutamato causa a despolarização sustentada de neurônios locais, que por sua vez, desencadeiam uma seqüência de eventos intracelulares que culminam no influxo de $Na^+$ e $Ca^{2+}$ e a exocitose adicional de glutamato (Choi, 1994). A excitotoxicidade pode ser dividida em duas fases distintas: uma fase aguda na qual acontece uma intensa entrada de $Na^+$ extracelular acompanhada pelo influxo passivo de água e $Cl^-$ levando a um inchaço celular e a e morte celular do tipo necrótica, e uma segunda fase tardia em que ocorre uma entrada intensa de $Ca^+$ extracelular levando a morte neuronal que pode ocorrer várias horas após o início do estímulo (Choi, 1994). Os receptores não-NMDA parecem estar mais vinculados à fase precoce, enquanto os receptores NMDA são mais responsáveis pela deflagração da fase tardia (Choi, 1992). Estudos com antagonistas do sistema glutamatérgico sugerem um maior envolvimento dos receptores NMDA como

mediadores da morte neuronal associada com a exposição breve e intensa ao glutamato do que os outros tipos de receptores (AMPA e KA) (Walton e Dodd, 2007).

Existem vários mecanismos propostos para explicar a morte celular induzida por glutamato, a maioria deles inclui o aumento na concentração de $Ca^{2+}$ citosólico devido a uma superestimulação dos receptores glutamatérgicos (Trotti e cols., 1998; Danbolt, 2001; Arundine e Tymianski, 2003). Uma via citotóxica que é ativada como resultado da sobrecarga de $Ca^{2+}$, leva à ativação contínua de uma grande variedade de enzimas dependentes de $Ca^{2+}$, tais como lipases, proteases, endonucleases e outras enzimas catabólicas que, coletivamente, têm consequências prejudiciais para a função da célula, a estrutura da membrana e do citoesqueleto, e que finalmente, levam à morte celular (Sattler e Tymianski, 2000; Arundine e Tymianski, 2003). Uma segunda via citotóxica do $Ca^{2+}$ conduz à geração de EROs através da ativação da óxido nítrico sintase (NOS) e a formação excessiva de NO. Níveis elevados de $Ca^{2+}$ na mitocôndria desacoplam a fosforilação oxidativa, o que leva a uma diminuição do abastecimento de energia e ao aumento da produção de EROs (Choi, 1987; Bittigau e Ikonomidou, 1997). A produção excessiva de EROs leva a oxidação das membranas biológicas e a morte celular (Gotz e cols., 1994; Schulz e cols., 1995).

Sabe-se que as EROs são capazes de inibir a captação de glutamato em culturas de astrócitos (Gotz e cols., 1994) e essa inibição acontece através da oxidação de grupos tióis na estrutura dos transportadores (Volterra e cols., 1994). A inibição dos transportadores de glutamato por meio de oxidação de grupos tióis é consistente com a descoberta de que a captação de glutamato é regulada pela química do estado redox de resíduos de cisteína reativos na estrutura do transportador (Trotti e cols., 1997a; Trotti e cols., 1997b). A inibição da captação de glutamato pela oxidação de grupos tióis nos transportadores leva ao maior acúmulo de glutamato na fenda sináptica. Dessa forma, estes eventos poderiam desencadear um ciclo vicioso de cascatas neurotóxicas, a medida que o aumento do glutamato extracelular irá promover um aumento adicional da formação de EROs (Trotti e cols., 1998).

### 1.2.4. O papel da guanosina na modulação do sistema glutamatérgico

Os derivados de guanina têm sido tradicionalmente estudados como moduladores de processos intracelulares, principalmente em relação a atividade de proteínas G na transdução de sinais (Schmidt e cols., 2007). No entanto, elas também exercem vários efeitos extracelulares não relacionados com a modulação de proteínas G, incluindo a modulação da atividade glutamatérgica, efeitos comportamentais, e efeitos tróficos sobre as células neurais (Baron e cols., 1989; Souza e Ramirez, 1991; Paz e cols., 1994; Ramos e cols., 1997; Burgos

e cols., 1998; Burgos e cols., 2000; Roesler e cols., 2000; Schmidt e cols., 2000; Ciccarelli e cols., 2001; Lara e cols., 2001; Tasca e cols., 2004; Vinade e cols., 2005).

Estudos têm demonstrado que a guanosina, um nucleosídeo de guanina, pode exercer efeitos tróficos em células neurais bem como modular o sistema glutamatérgico (Rathbone e cols., 1999; Ciccarelli e cols., 2001; Soares e cols., 2004). Os mecanismos envolvidos nestes efeitos parecem estar relacionados com a estimulação sobre a captação de glutamato conferida pela guanosina (Frizzo e cols., 2001; Frizzo e cols., 2002; Frizzo e cols., 2003; Schmidt e cols., 2005). De fato a guanosina exibiu efeitos protetores em uma variedade de modelos de neurotoxicidade envolvendo a superativação de receptores glutamatérgicos tanto *in vitro*, quanto *in vivo* (Frizzo e cols., 2001). Em modelos *in vitro*, a guanosina inibiu a ligação do glutamato e de seus análogos, preveniu respostas celulares aos aminoácidos excitatórios, estimulou a captação de glutamato extracelular por culturas de astrócitos e fatias de cérebro, e protegeu fatias de cérebro expostas a hipóxia/ hiperglicemia (Schmidt e cols., 2005). Em modelos *in vivo*, a guanosina protegeu contra convulsões induzidas por agentes glutamatérgicos em roedores (Schmidt e cols., 2005). Dessa forma, as evidências apontam que os derivados de guanina, como a guanosina, são potenciais alvos terapêuticos na neuroproteção e na neuromodulação (Schmidt e cols., 2007).

### 1.3. Metilmercúrio

O MeHg é uma forma orgânica de mercúrio encontrada no ambiente aquático devido a fontes de origem natural e antropogênica. O MeHg é gerado através da biometilação do mercúrio inorgânico presente em sedimentos aquáticos por bactérias redutoras de sulfato (Clarkson e cols., 2003). Este processo leva ao subsequente acúmulo de MeHg na cadeia alimentar de organismos aquáticos, alcançando suas maiores concentrações em peixes predadores (Clarkson e cols., 2003). O MeHg apresenta um grande potencial de biomagnificação podendo acumular em mais de sete ordens de magnitude a partir de concentrações sub ng/L na água para concentrações superiores a 1 mg/kg em peixes piscívoros (Hintelmann, 2010). Em consequência disso, peixes contaminados constituem a principal fonte de exposição ao MeHg em humanos (Clarkson e cols., 2003).

Embora, a toxicidade do MeHg seja conhecida a bastante tempo, ela só passou a ser mais profundamente estudada após incidentes catastróficos de intoxicação por este metal decorrentes do consumo de alimentos contaminados ocorridos entre as décadas de 50 e 70 (Hunter e Russell, 1954; Takeuchi e cols., 1962; Bakir e cols., 1973; Davis e cols., 1994). A liberação industrial de MeHg na Baía de Minamata e no rio Agano, Japão, entre os anos 1950

e 1960 afetou milhares de pescadores e suas famílias os quais passaram a sofrer de uma doença neurológica, exibindo sinais de descoordenação motora, redução dos campos visuais e dormência nas extremidades (Harada, 1978).

Anos mais tarde, surtos de intoxicação por MeHg ocorreram em vários países e foram causados pela preparação caseira de pão diretamente a partir de grãos tratados com um fungicida que continha MeHg (WHO, 1976.). O mais grave desses surtos ocorreu no Iraque entre 1971 e 1972 onde houve cerca de 6000 casos de internação em hospitais, e 459 mortes registradas (Bakir e cols., 1973).

Os episódios mencionados contribuíram para o entendimento dos principais sintomas clínicos bem como alterações histológicas provocados pela intoxicação com MeHg em humanos, todavía, os mecanismos moleculares envolvidos na toxicidade do MeHg ainda não foram totalmente elucidados. Além disso, não existe até o presente momento um tratamento eficaz contra a intoxicação por MeHg e, portanto, o estudo da toxicologia do MeHg é de grande importância uma vez que pode ajudar na busca por novas terapias para o tratamento da intoxicação por este contaminante ambiental.

### 1.3.1 Absorção, distribuição e excreção

Cerca de 95% do MeHg ingerido é absorvido no trato gastrointestinal, embora o local exato de absorção não seja conhecido. Ele é distribuído a todos os tecidos em um processo concluído em cerca de 30 horas, sendo que, aproximadamente 5% do MeHg é encontrado no sangue e cerca de 10% no cérebro (Clarkson e cols., 2003). O MeHg é lentamente metabolizado em mercúrio inorgânico principalmente pela microflora nos intestinos, provavelmente a uma taxa de cerca de 1% da carga corporal por dia. Embora o MeHg seja a forma predominante de mercúrio durante a exposição, o mercúrio inorgânico lentamente acumula e reside por longos períodos no SNC. Acredita-se que seja uma forma inerte, provavelmente insolúvel de seleneto de mercúrio (WHO, 1990.).

A excreção urinária de MeHg é da ordem de 10%, ou menos, da eliminação total do organismo. O MeHg passa por um extenso ciclo entero-hepático. Ele é secretado na bile e parcialmente reabsorvido pela circulação portal, assim, retornando para o fígado. Uma fração do mercúrio biliar é convertida pela microflora em mercúrio inorgânico, o qual é reabsorvido em uma pequena escala. Assim, a maior parte do MeHg é eliminado por demetilação e excreção da forma inorgânica nas fezes (Clarkson e cols., 2003).

O MeHg está presente no organismo na forma de complexos solúveis em água principalmente ligado a grupos tióis. Ele atravessa a barreira hemato-encefálica como um

complexo com a L-cisteína. O complexo MeHg-L-cisteína é estruturalmente semelhante ao aminoácido L-metionina, e consequentemente pode atravessar as membranas celulares via transportador de aminoácidos neutros (Kerper e cols., 1992). O MeHg é bombeado para fora das células na forma de um complexo principalmente com o GSH. Este complexo é secretado na bile e, então a molécula de GSH é degradada no ducto biliar e vesícula biliar em um dipeptídeo e, finalmente, no complexo L-cisteína. Presumivelmente, nesta forma o MeHg é reabsorvido na corrente sanguínea para ser retornado ao fígado, completando assim o ciclo entero-hepático (Ballatori e Clarkson, 1985; Dutczak e Ballatori, 1992, 1994).

### 1.3.2. Toxicidade

O SNC é o alvo principal dos efeitos tóxicos do MeHg, em particular, o SNC em desenvolvimento. Mesmo uma exposição a baixas doses de MeHg pode ter consequências adversas para o SNC, especialmente durante a vida pré-natal levando a atrasos no desenvolvimento cognitivo. Os problemas neurológicos decorrentes da exposição ao MeHg incluem: déficits de coordenação motora, fraqueza e atrofia muscular, e diminuição ou perda progressiva das funções sensoriais incluindo prejuízos da fala, visão e audição (Harada, 1997; Limke e cols., 2004). Além do SNC, o fígado e os rins parecem ser órgãos alvo importantes para a toxicidade do MeHg (Mottet e cols., 1985; Zalups, 2000; Diaz e cols., 2001; Bragadin e cols., 2002; Farina e cols., 2004; Flora e cols., 2008).

#### 1.3.2.1. Mecanismos de toxicidade

A toxicidade do MeHg tem sido atribuída a três mecanismos principais: a sua interação com grupos -SH e selenoidrila (SeH) formando complexos com compostos contendo tiol e selenol (Clarkson, 1972; Farina e cols., 2011), o disturbio dos níveis intracelulares de $Ca^{2+}$ (Atchison e Hare, 1994; Graff e cols., 1997) e, a indução de estresse oxidativo pela superprodução de EROs ou pela redução na capacidade de defesa antioxidante da célula (Lebel e Schatz, 1990; Sarafian e Verity, 1991; Yee e Choi, 1994).

O MeHg possui uma alta afinidade por grupamentos tiol e selenol podendo se ligar a uma variedade de biomoléculas de baixo e alto peso molecular, o que é responsável, pelo menos em parte, pela redução da capacidade antioxidante e o aumento da geração de EROs induzida pelo MeHg (Kaur e cols., 2006; Franco e cols., 2007; Farina e cols., 2009; Aschner e cols., 2010; Farina e cols., 2011b). Isso se deve, principalmente ao fato de que o MeHg pode prejudicar a atividade de proteínas contendo tiol e selenol, como a GPx, a Trx e a TrxR (Carvalho e cols., 2008; Farina e cols., 2009; Franco e cols., 2009; Glaser e cols., 2010a;

Wagner e cols., 2010a; Branco e cols., 2011; Carvalho e cols., 2011). Estas protéinas são componentes importantes do sistema antioxidante celular, e a sua inibição contribui para a ruptura do equilíbrio redox normal das células (Farina e cols., 2011). Além disso, a ligação do MeHg ao GSH, devido à sua elevada afinidade por grupos -SH, diminui a disponibilidade deste antioxidante, expondo as células aos danos mediados por radicais livres (Shanker e cols., 2005).

A GSH é o tiol de baixo peso molecular, mais importante e abundante nas células de mamíferos. Além das suas propridades antioxidantes, os efeitos protetores do GSH estão relacionados também à sua capacidade de complexar com o mercúrio limitanto a quantidade deste metal disponível para a interação com macromoléculas sensíveis. Dessa forma, o fornecimento de precursores de GSH aos neurônios via astrócitos e a manutenção das concentrações intracelulares de GSH são cruciais para proteger as células contra a neurotoxicidade induzida pelo MeHg. O MeHg é conhecido por inibir o transporte de cistina, o precursor chave da síntese de GSH, via transportadores $X_{AG}$- em astrócitos. O subseqüente esgotamento dos níveis de GSH em células neurais aumenta a vulnerabilidade das células ao MeHg (Shanker e Aschner, 2001; Allen e cols., 2002).

Estudos *in vitro* demonstraram que o MeHg é capaz de inibir a captação de glutamato em culturas de astrócitos (Porciuncula e cols., 2003). O MeHg também aumentou a liberação de glutamato em vesículas sinápticas e fatias corticais de cérebro de rato (Moretto e cols., 2005). Esses eventos levariam ao aumento dos níveis extracelulares de glutamato que como visto anteriormente desencadeia uma cascata de eventos culminando na morte neuronal.

O aumento do $Ca^{2+}$ intracelular foi demonstrado em vários tipos celulares após a exposição ao MeHg (Sarafian, 1993; Atchison e Hare, 1994; Graff e cols., 1997; Marty e Atchison, 1997, 1998). Em células granulares do cerebelo, as alterações parecem estar relacionadas inicialmente com a mobilização de $Ca^{2+}$ a partir das reservas intracelulares (primeira fase), seguido da entrada de $Ca^{2+}$ através de canais de membrana (segunda fase) (Marty e Atchison, 1997). Efeitos como o aumento dos níveis de inositol fosfato e alterações da fosforilação de proteínas, possivelmente devido à ação de níveis elevados de $Ca^{2+}$ como um segundo mensageiro, também têm sido descritos (Saijoh e cols., 1993; Sarafian, 1993).

A liberação descontrolada de $Ca^{2+}$ da mitocôndria também foi relatada durante o estresse oxidativo, uma condição que está envolvida na toxicidade do MeHg (Ali e cols., 1992; Yee e Choi, 1996; Sarafian, 1999; Sanfeliu e cols., 2001). De fato, diversos trabalhos têm demonstrado o aumento dos níveis de EROs após a exposição ao MeHg em vários tecidos (Taylor e cols., 1973; Yonaha e cols., 1983; Lebel e cols., 1990; Sarafian e Verity, 1991;

InSug e cols., 1997; Shenker e cols., 1999; Mundy e Freudenrich, 2000; Oyama e cols., 2000; Farina e cols., 2011a).

O aumento dos níveis de EROs induzido por MeHg também pode estar relacionado a alterações nas funções mitocondriais. As mitocôndrias são as principais organelas envolvidas na produção de EROs e também um dos sítios mais sucetíveis ao dano induzido por EROs (Mori e cols., 2007). Entre as consequências do estresse oxidativo induzido por MeHg está a abertura do poro de transição de permeabilidade mitocondrial (PTPM), um processo dependente de $Ca^{2+}$ (Aschner e cols., 2007). Este evento causa o aumento da permeabilidade a prótons, íons e outros solutos $\leq$ 1500 Da, levando ao colapso do potencial de membrada da mitocôndria ($\Delta\Psi m$). A perda do $\Delta\Psi m$ resulta no inchaço osmótico da matriz mitocondrial, movimento de metabólitos através da membrana interna, fosforilação oxidativa deficiente, interrupção da síntese de ATP, geração de EROs e morte celular (Atchison e Hare, 1994; Aschner e cols., 2007).

### 1.4. Justificativa

Diversos estudos têm demonstrado o envolvimento do estresse oxidativo no desenvolvimento de doenças neurodegenerativas ou resultantes da exposição à contaminantes ambientais potencialmente neurotóxicos. Dessa forma, compostos antioxidantes que possuam atividades biológicas e ações farmacológicas têm uma aplicação importante como neuroprotetores. Em vista disto, compostos orgânicos de selênio com propriedades antioxidantes vêm sendo sintetizados e estudados devido ao seu potencial como agentes terapêuticos e, portanto, o seu estudo frente à agentes pró-oxidantes potencialmente neurotóxicos é de grande importância. Além disso, a toxicologia dos compostos orgânicos de selênio também precisa ser melhor estudada uma vez que trabalhos têm demonstrado que em doses altas estes compostos podem apresentar efeitos tóxicos em roedores.

## 1.5. Objetivos

### 1.5.1. Objetivo Geral

O presente estudo teve como objetivo geral investigar os efeitos dos compostos orgânicos de selênio, ebselen e disseleneto de difenila, frente à toxicidade induzida por glutamato ou MeHg.

### 1.5.2. Objetivos Específicos

Artigo 1

1- Avaliar os efeitos dos compostos orgânicos de selênio ebselen e disseleneto de difenila sobre a produção de EROs induzida por glutamato em fatias de córtex, estriado e hipocampo de rato;
2- Avaliar os efeitos da guanosina, um modulador do sistema glutamatérgico, sobre a produção de EROs induzida por glutamato em fatias de córtex, estriado e hipocampo de rato;
3- Testar os efeitos da guanosina sobre a inibição da captação de [$^3$H]-glutamato em fatias de córtex, estriado e hipocampo de rato;
4- Investigar os efeitos das interações entre compostos que modulam a atividade do sistema glutamatérgico (guanosina) e antioxidantes (ebselen e disseleneto de difenila) sobre a produção de EROs induzida por glutamato em fatias de córtex, estriado e hipocampo de rato;

Manuscrito 1

1- Avaliar os efeitos da exposição ao disseleneto de difenila e/ ou MeHg na produção de EROs em mitocôndrias isoladas de fatias de fígado de rato;
2- Verificar os efeitos da exposição ao disseleneto de difenila e/ ou MeHg sobre a capacidade de redução do MTT (atividade de enzimas desidrogenases) de mitocôndrias isoladas de fatias de fígado de rato;
3- Avaliar os efeitos da exposição ao disseleneto de difenila e/ ou MeHg sobre o $\Delta\psi m$ de mitocôndrias isoladas de fatias de fígado de rato;
4- Estudar os efeitos da exposição ao disseleneto de difenila e/ ou MeHg sobre o consumo de oxigênio por mitocôndrias isoladas de fatias de fígado de rato;

Manuscrito 2

1- Investigar os efeitos do tratamento com disseleneto de difenila sobre alterações na atividade motora induzidas por MeHg em ratos;
2- Avaliar os efeitos do tratamento com disseleneto de difenila e/ ou MeHg em diferentes indicadores de função mitocondrial em cérebro e fígado de rato;
3- Avaliar os efeitos do tratamento com disseleneto de difenila e/ ou MeHg sobre a atividade da enzima TrxR em cérebro, fígado e rins de rato;
4- Estudar os efeitos do tratamento com disseleneto de difenila sobre a deposição de mercúrio em cérebro, fígado e músculo esquelético de rato.

## 2. ARTIGOS CIENTÍFICOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos científicos, os quais se encontram aqui organizados. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se no artigo e nos manuscritos. O **artigo 1** está disposto na forma em que foi publicado na revista Brain Research e os **manuscritos 1** e **2** estão dispostos na forma em que normalmente se submete para publicação.

## 2.1. – A COMBINAÇÃO DE COMPOSTOS ORGÂNICOS DE SELÊNIO E GUANOSINA PREVINE O ESTRESSE OXIDATIVO INDUZIDO POR GLUTAMATO EM DIFERENTES REGIÕES DO CÉREBRO DE RATO

**Artigo 1**

**THE COMBINATION OF ORGANOSELENIUM COMPOUNDS AND GUANOSINE PREVENTS GLUTAMATE-INDUCED OXIDATIVE STRESS IN DIFFERENT REGIONS OF RAT BRAINS**

CRISTIANE L. DALLA CORTE, LUÍZA L. BASTOS, FERNANDO DOBRACHINSKI, JOÃO B.T. ROCHA, FÉLIX A.A. SOARES

(Publicado na revista Brain Research)

ARTICLE IN PRESS

BRES-41876; No. of pages: 11; 4C:

BRAIN RESEARCH XX (2011) XXX–XXX

Available online at www.sciencedirect.com

SciVerse ScienceDirect

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

# The combination of organoselenium compounds and guanosine prevents glutamate-induced oxidative stress in different regions of rat brains

*Cristiane L. Dalla Corte, Luíza L. Bastos, Fernando Dobrachinski, João B.T. Rocha, Félix A.A. Soares**

*Departamento de Química, Programa de Pós-graduação em Ciências Biológicas, Bioquímica Toxicológica, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Cep 97105-900, Santa Maria, RS, Brazil*

ARTICLE INFO

*Article history:*
Accepted 28 October 2011

*Keywords:*
Glutamate
Organoselenium compound
Guanosine
Oxidative stress
Excitotoxicity

ABSTRACT

This study was designed to investigate the protective effects of the combination of guanosine and 2 organoselenium compounds (ebselen and diphenyl diselenide) against glutamate-induced oxidative stress in different regions of rat brains. Glutamate caused an increase in reactive oxygen species (ROS) generation and a decrease in [$^3$H]-glutamate uptake in striatal, cortical, and hippocampal slices. Guanosine, ebselen, and diphenyl diselenide prevented glutamate-induced ROS production in striatal, cortical and hippocampal slices. The combination of guanosine with organoselenium compounds was more effective against glutamate-induced ROS production than the individual compounds alone. Guanosine prevented [$^3$H]-glutamate uptake inhibition in striatal, cortical, and hippocampal slices. Thus, protection against the harmful effects of glutamate is possibly due to the combination of the antioxidant properties of organoselenium compounds and the stimulatory effect of guanosine on glutamate uptake. In conclusion, the combination of antioxidants and glutamatergic system modulators could be considered a potential therapy against the prooxidant effects of glutamate.



## 1. Introduction

The amino acid L-glutamate is the predominant excitatory neurotransmitter in the central nervous system (CNS) of mammals (Gilgun-Sherki et al., 2002; McEntee and Crook, 1993). After being released in the synaptic cleft, L-glutamate must be removed quickly and efficiently from the extracellular fluid by high-affinity transporters to avoid receptor overstimulation (Danbolt, 2001; Trotti et al., 1998; Walton and Dodd, 2007). The glutamate transporters in the plasma membranes of both astrocytes and neurons perform this task (Trotti et al., 1998). These glutamate transporters have an important role in terminating synaptic transmission, as sustained elevation of extracellular glutamate triggers neuronal death through a mechanism termed excitotoxicity (Choi, 1992; Mejia-Toiber et al., 2006).

* *Corresponding author*. Fax: +55 55 3220 8978.
E-mail address: felix@ufsm.br (F.A.A. Soares).

*Abbreviations:* ROS, reactive oxygen species; CNS, central nervous system; GPx, glutathione peroxidase; TBARS, thiobarbituric acid-reactive species; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase; DCFH, 2′,7′-dichlorofluorescin diacetate; NO, nitric oxide; ACSF, artificial cerebrospinal fluid; TBA, thiobarbituric acid; MDA, malondialdehyde; DMSO, dimethylsulfoxide; NAD+, β-nicotinamide adenine dinucleotide; NADH, reduced β-nicotinamide adenine dinucleotide

0006-8993/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2011.10.049

Excitotoxicity has been implicated in several neuronal disorders such as epilepsy, ischemia, trauma, amyotrophic lateral sclerosis, Huntington's disease, and Alzheimer's disease (Walton and Dodd, 2007, and references therein). The mechanisms responsible for the elevation of extracellular glutamate include enhanced release of glutamate and the reduction of glutamate uptake (Gilgun-Sherki et al., 2002). This process appears to involve sustained elevations of intracellular calcium levels through glutamate transporters, leading to an imbalance of sodium ions across plasma membranes (Gilgun-Sherki et al., 2002). Excitatory events may stimulate ROS production, and there is evidence that ROS can promote additional glutamate release, generating a vicious amplifying cycle of the neurotoxic cascade (Mailly et al., 1999; Pellegrini-Giampietro et al., 1990). Indeed, the vulnerability of glutamate transporters to oxidants provides evidence that excitotoxicity and oxidative stress are interrelated and act synergistically to induce neuronal damage (Coyle and Puttfarcken, 1993; Gilgun-Sherki et al., 2002; Trotti et al., 1998). This concept has general therapeutic implications, as it suggests that using anti-excitotoxic and antioxidant agents in combination may improve neuroprotection (Trotti et al., 1998).

The redox activity of selenium-containing molecules has been suggested to provide antioxidants of considerable potency (Nogueira et al., 2004; Parnham and Graf, 1991). This has stimulated interest in the synthesis and reactivity of organoselenium compounds, with particular emphasis on their biological properties (Engman et al., 1997; Mugesh et al., 2001; Nogueira et al., 2004). Of particular importance is that the antioxidant activity of various organoselenium compounds appears to be related, at least in part, to their glutathione peroxidase (GPx)-like activity (Mugesh et al., 2001; Nogueira et al., 2004).

Ebselen was the first organoselenium compound suggested for hydroperoxide-inactivating therapy in the presence of glutathione (Nogueira et al., 2004). Ebselen has been safely administered in several animal models of brain injuries with pronounced neuroprotective effects (Namura et al., 2001; Takasago et al., 1997). Based on the encouraging evidence of the neuroprotective effects of ebselen in animal models, clinical trials were conducted with this compound, obtaining modest success (Gilgun-Sherki et al., 2002; Yamaguchi et al., 1998). Diphenyl diselenide (the simplest of the diaryl diselenides) has been demonstrated to be even more active as a GPx mimic and less toxic to rodents than ebselen (Meotti et al., 2003; Wilson et al., 1989). In fact, diphenyl diselenide has a protective role in a variety of *in vitro* and *in vivo* experimental models associated with the overproduction of free radicals in the brain, liver, and kidney (Burger et al., 2004; Burger et al., 2006; Funchal et al., 2006; Ghisleni et al., 2003, 2008; Kade et al., 2009; Nogueira et al., 2004; Stangherlin et al., 2008).

Several studies have demonstrated that the extracellular nucleoside guanosine can exert trophic effects on neural cells, as well as modulate the glutamatergic system (Ciccarelli et al., 2001; Rathbone et al., 1999; Soares et al., 2004). The mechanism involved in these effects appears to be related to a stimulatory effect on glutamate uptake afforded by guanosine (Frizzo et al., 2001, 2002, 2003; Halliwell, 2006; Margaill et al., 2005; Schmidt et al., 2005). Indeed, guanosine exhibits protective effects in a variety of *in vitro* and *in vivo* models of neurotoxicity that involve the overactivation of glutamate receptors (Frizzo et al., 2001). In *in vitro* models, guanosine inhibited glutamate and analogous binding, prevented cell responses to excitatory amino acids, stimulated the uptake of extracellular glutamate by astrocyte cultures and brain slices, and protected brain slices exposed to hypoxia/hypoglycemia (Schmidt et al., 2005, and references therein). In *in vivo* models, guanosine protected against seizures induced by glutamatergic agents in rodents (Schmidt et al., 2005).

Although antioxidants and purine derivatives are very promising compounds for the treatment of CNS disorders, there is no efficient curative treatment that has been proven effective to date. Thus, combinations of treatments may have synergistic effects that are more successful. In the present study, we investigated the protective effects of the combination of guanosine and 2 organoselenium compounds (ebselen and diphenyl diselenide) against glutamate-induced oxidative stress.

## 2. Results

### 2.1. *Lipid peroxidation*

Thiobarbituric acid-reactive substance (TBARS) levels in cortical and hippocampal slices treated with 10 mM glutamate were significantly higher than that observed in control slices ($p<0.05$) (Table 1). TBARS levels in striatal slices were not affected by treatment with any concentration of glutamate (Table 1).

### 2.2. *Cellular viability*

Cellular viability was evaluated by measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a formazan product by mitochondrial dehydrogenases. Glutamate was not able to decrease mitochondrial viability at any concentration tested in striatal, cortical, and hippocampal slices (data not shown). Lactate dehydrogenase (LDH) release can be an index of cytotoxicity, as it is indicative of disruption of the cellular membrane. Glutamate did not alter LDH release at any concentration tested in striatal, cortical, and hippocampal slices (data not shown).

### 2.3. *ROS generation*

#### 2.3.1. *Glutamate × guanosine*

Glutamate significantly increased ROS production (2′,7′-dichlorofluorescin diacetate (DCFH) oxidation) in striatal,

**Table 1 – Effect of glutamate on lipid peroxidation.**

| Glutamate (mM) | TBARS production (% control) | | |
|---|---|---|---|
| | Striatum | Cortex | Hippocampus |
| 0 | 100±8.2 | 103.4±1.7 | 96±4.2 |
| 0.01 | 81.5±5.4 | 93.9±1.9 | 117.5±4.2 |
| 0.1 | 96±1.6 | 100.9±3.8 | 101.2±0.8 |
| 1 | 100.4±5.8 | 102.5±2.9 | 125±5.9 |
| 10 | 104.1±4.4 | 134.5±11.6* | 148.7±18.1* |

Data are mean±SEM for n=4–5 in each group.
* $p<0.05$ compared to control.

Please cite this article as: Dalla Corte, C.L., et al., The combination of organoselenium compounds and guanosine prevents glutamate-induced oxidative stress in different regions of rat brains, Brain Res. (2011), doi:10.1016/j.brainres.2011.10.049

cortical, and hippocampal slices at concentrations of 1 and 10 mM ($p<0.05$, Fig. 1). Guanosine (100 μM) prevented ROS production induced by 1 mM glutamate in striatal, cortical, and hippocampal slices, but could not completely prevent ROS production when it was induced by 10 mM glutamate ($p<0.05$, Fig. 1). Different concentrations of guanosine were used to prevent DCFH oxidation induced by 1 mM glutamate. Treatment with 100 and 200 μM guanosine blunted glutamate (1 mM)-induced ROS production by striatal slices ($p>0.05$, Fig. 2A). In cortical and hippocampal slices, guanosine treatment prevented ROS production at concentrations of 10 and 20 μM, respectively ($p<0.05$, Figs. 2B, C). Cortical and hippocampal slices were more sensitive to guanosine than striatal slices were.

### 2.3.2. *Glutamate × organoselenium compounds*

At low concentrations, diphenyl diselenide and ebselen significantly prevented glutamate (1 mM)-induced ROS generation in

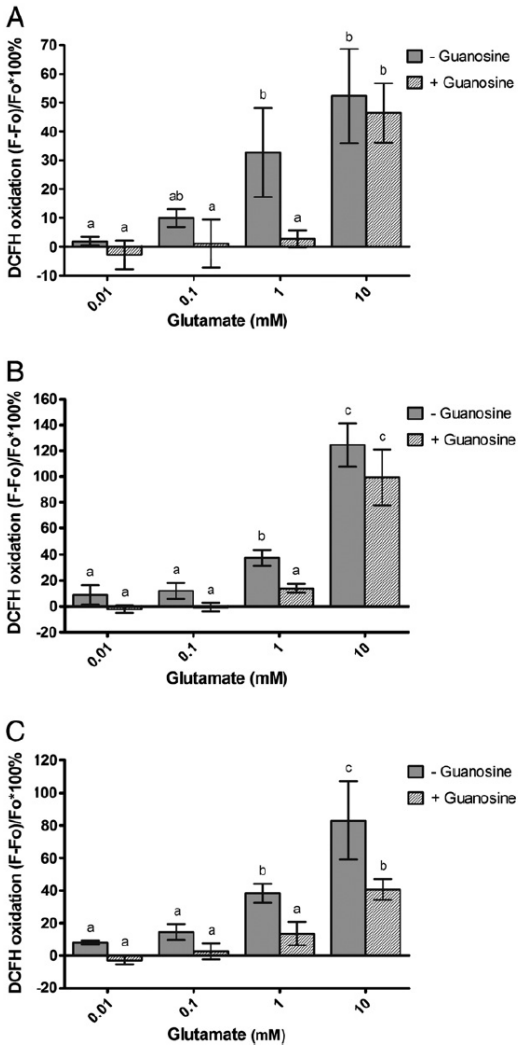



**Fig. 1 – Effect of guanosine (100 μM) and glutamate on ROS production in striatal (A), cortical (B), and hippocampal (C) slices from rats. Data are expressed as the mean ± S.E.M., n = 6. Experiments were performed in duplicate. Bars with different letters are significantly different ($p<0.05$) from each other. Bars with the letter a are statistically indistinguishable from the control.**

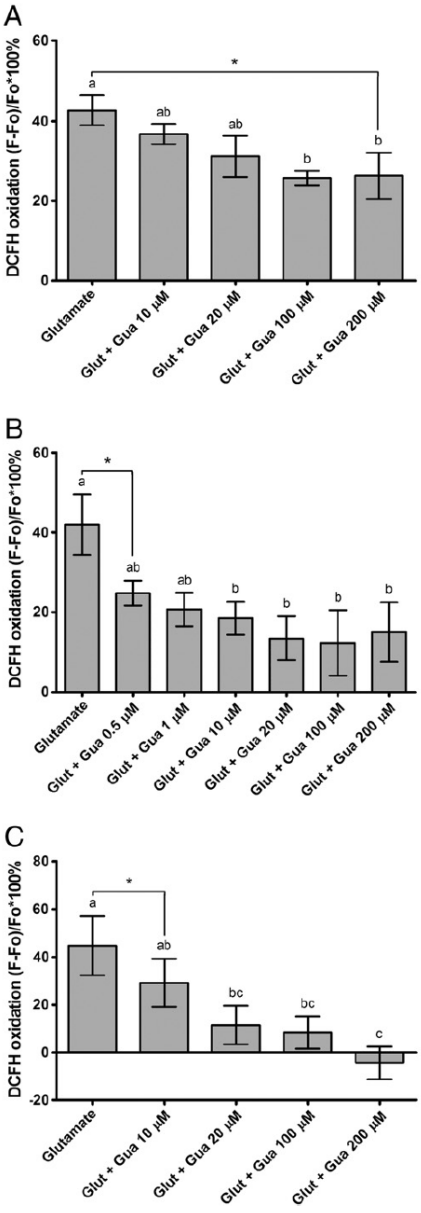
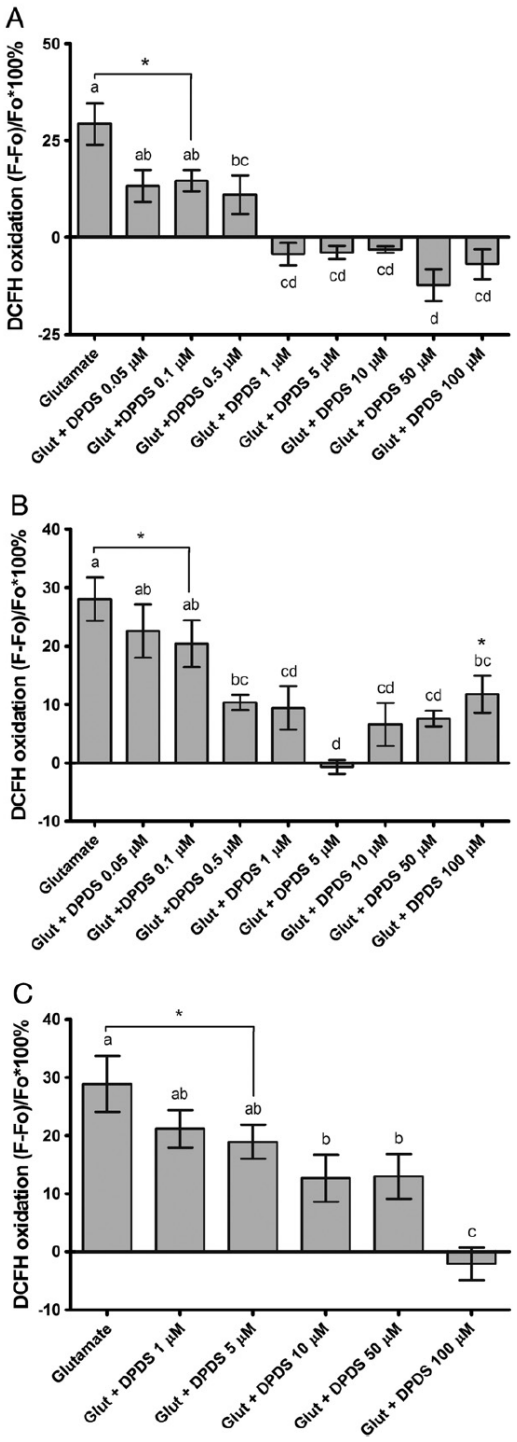



**Fig. 2 – Effect of guanosine (Gua) on glutamate (Glut)-induced ROS production in striatal (A), cortical (B), and hippocampal (C) slices from rats. Data are expressed as the mean ± S.E.M., n = 6. Experiments were performed in duplicate. Bars with different letters are significantly different ($p<0.05$) from each other. Asterisk indicates significant differences from the control ($p<0.05$).**

Please cite this article as: Dalla Corte, C.L., et al., The combination of organoselenium compounds and guanosine prevents glutamate-induced oxidative stress in different regions of rat brains, Brain Res. (2011), doi:10.1016/j.brainres.2011.10.049

striatal, cortical, and hippocampal slices. Treatment with diphenyl diselenide decreased glutamate (1 mM)-induced ROS production to the control levels at concentrations of 0.5, 0.5, and 10 μM in striatal, cortical, and hippocampal slices, respectively ($p<0.05$, Fig. 3). Ebselen prevented glutamate (1 mM)-induced ROS production at concentrations of 0.5, 1, and 5 μM in striatal, cortical, and hippocampal slices, respectively ($p<0.05$, Fig. 4).




### 2.3.3. *Glutamate × combined treatment (guanosine + organoselenium compounds)*

Considering that organoselenium compounds act as antioxidants and guanosine could affect glutamate uptake, the use of both in combination could better protect against glutamate induced-ROS generation, as they could act via different mechanisms. To test this hypothesis, we investigated whether noneffective concentrations of each compound in combination could exert protective effects against glutamate induced-ROS generation to avoid any possible toxic effect caused by high concentrations of these compounds. Indeed, the combination of 10 μM guanosine and 1 μM diphenyl diselenide was more effective in preventing glutamate-induced ROS generation in striatal, cortical, and hippocampal slices than the individual compounds alone ($p<0.05$, Fig. 5). The combination of 100 μM guanosine with diphenyl diselenide (1 μM) was also effective in protecting against glutamate-induced ROS production in striatal, cortical, and hippocampal slices, although this protection was not different from that exerted by guanosine (100 μM) and diphenyl diselenide (1 μM) alone ($p<0.05$, Fig. 5).

The combination of ebselen (1 μM) and guanosine (10 μM) was better than the individual compounds alone in protecting against ROS generation induced by glutamate (1 mM) in striatal, cortical, and hippocampal slices ($p<0.05$, Fig. 6). Treatment of striatal, cortical, and hippocampal slices with the combination of guanosine (100 μM) and ebselen (1 μM) significantly prevented glutamate (1 mM)-induced ROS production, although these changes were indistinguishable from those caused by either compound [guanosine (100 μM) and ebselen (1 μM)] alone ($p<0.05$, Fig. 6).

### 2.4. *[$^3$H]-Glutamate uptake*

Glutamate (1 mM) inhibited [$^3$H]-glutamate uptake in striatal (44.7% inhibition), cortical (24.9% inhibition), and hippocampal (41.4% inhibition) slices compared to the intake in control slices ($p<0.05$, Fig. 7). Treatment with 10 and 100 μM guanosine prevented the inhibition of [$^3$H]-glutamate uptake in striatal, cortical, and hippocampal slices ($p<0.05$, Fig. 7).

## 3. Discussion

The overstimulation of glutamate receptors triggers a sequence of cellular events that includes oxidative stress and contributes to the pathophysiology of a number of brain injuries (Danbolt, 2001; Mejia-Toiber et al., 2006; Walton and Dodd, 2007). In this

**Fig. 3 – Effect of diphenyl diselenide (DPDS) on glutamate (Glut)-induced ROS production in striatal (A), cortical (B), and hippocampal (C) slices from rats. Data are expressed as the mean ± S.E.M., n = 6. Experiments were performed in duplicate. Bars with different letters are significantly different ($p<0.05$) from each other. Asterisk indicates significant differences from the control ($p<0.05$).**

Please cite this article as: Dalla Corte, C.L., et al., The combination of organoselenium compounds and guanosine prevents glutamate-induced oxidative stress in different regions of rat brains, Brain Res. (2011), doi:10.1016/j.brainres.2011.10.049

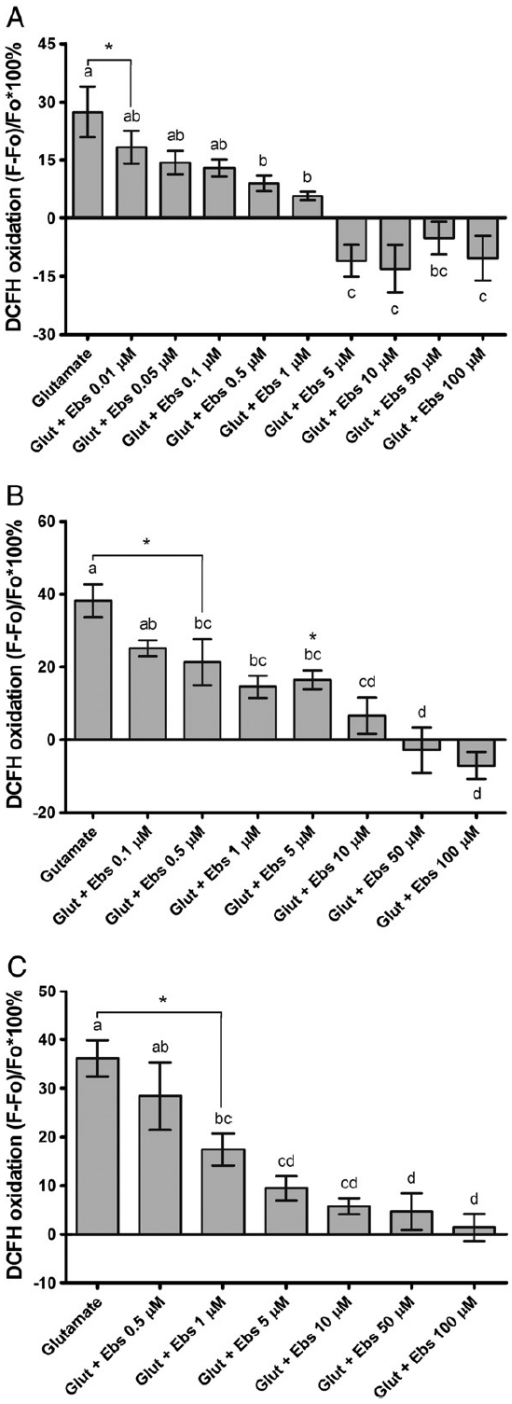



**Fig. 4 – Effect of ebselen (Ebs) on glutamate (Glut)-induced ROS production in striatal (A), cortical (B), and hippocampal (C) slices from rats. Data are expressed as the mean ± S.E.M., n = 6. Experiments were performed in duplicate. Bars with different letters are significantly different ($p < 0.05$) from each other. Asterisk indicates significant differences from the control ($p < 0.05$).**

study, glutamate augmented ROS production as well as decreased its uptake in striatal, cortical, and hippocampal slices from rats, characterizing the primary events of harmful glutamatergic overstimulation. Glutamate induced an increase in TBARS levels in cortical and hippocampal slices only at the highest concentration tested (10 mM); however, the concentration of 1 mM used throughout the study did not induce changes in the TBARS levels. Here, we would like to call attention to the fact that the TBARS assay is only a global test for assessing lipid peroxidation because thiobarbituric acid (TBA) is nonspecific for malondialdehyde (MDA) and can react with nonlipid-related materials and fatty peroxide-derived decomposition products other than MDA (Janero, 1990). In this manner, utilization of the TBARS assay in lipid peroxidation studies requires caution regarding the interpretation of its results. Glutamate did not induce a loss of viability in the assays performed in this study (MTT reduction and LDH release), indicating that the slices were viable during all experiments despite the increase in ROS levels and the impairment of glutamate uptake. In addition, we demonstrated that guanosine, ebselen, and diphenyl diselenide protected striatal, cortical, and hippocampal slices from rats from the ROS production evoked by glutamate.

An oxidative stress condition and impairment of glutamate transport have been observed in parallel in several brain pathologies leading to acute or chronic neurodegeneration (Trotti et al., 1998). Amyotrophic lateral sclerosis, Alzheimer's disease, and ischemic and traumatic injuries are examples of brain pathologies in which alterations in glutamate transport and oxidative stress are involved. The toxic pathways by which exposure to excess glutamate can lead to aberrant ROS formation include $Ca^{2+}$-dependent activation of nitric oxide (NO) synthase, phospholipase $A_2$, and xanthine oxidase (Trotti et al., 1998). In this manner, antioxidants are certainly a promising therapeutic class for the treatment of neurodegenerative diseases (Halliwell, 2006; Margaill et al., 2005).

Organoselenium compounds have been proposed as potential therapeutic agents against the formation of free radicals and the deleterious actions of ROS secondary to a variety of neuronal injuries (Centuriao et al., 2005; Nogueira et al., 2004; Posser et al., 2008; Zhao and Holmgren, 2002). In fact, the neuroprotective effects of ebselen and diphenyl diselenide have been attributed to the antioxidant properties of these compounds (Rossato et al., 2002; Warren, 2002). In addition, it has been reported that low selenium concentrations in the elderly were significantly associated with senility and cognitive decline (Berr et al., 2000). In view of these findings, the protective effects of both compounds could be related to their ability to prevent the first signs of glutamate neurotoxicity, such as oxidative stress.

In this study, guanosine was able to prevent the increase in ROS generation induced by glutamate in striatal, cortical, and hippocampal slices. These results again demonstrate the relationship between increased extracellular glutamate and increased ROS production, as guanosine also protected against the inhibition of glutamate uptake in all brain regions. In fact, guanine-based purines such as guanosine are well-known modulators of the glutamatergic system, and their neuroprotective effects have been demonstrated in several studies (Ciccarelli et al., 2001; Frizzo et al., 2001, 2002, 2003; Rathbone et al., 1999; Schmidt et al., 2005). Therefore, the protective effects of guanosine observed here could be related with its

Please cite this article as: Dalla Corte, C.L., et al., The combination of organoselenium compounds and guanosine prevents glutamate-induced oxidative stress in different regions of rat brains, Brain Res. (2011), doi:10.1016/j.brainres.2011.10.049

ability to reduce extracellular glutamate by activating glutamate uptake into astrocytes (Frizzo et al., 2002; Soares et al., 2004), subsequently preventing ROS production.

Although antioxidants and purine derivatives have proven efficiency in counteracting the toxic effects of glutamate, there are no effective therapeutics for CNS injuries associated with the overactivation of glutamate receptors to date. Thus, the combination of antioxidants and purine derivatives could be more interesting than each compound being used separately for 2 reasons. Firstly, it would afford protection via different mechanisms, as the antioxidant would act by attacking the ROS, whereas guanosine would act by modulating the glutamatergic system. Secondly, the combination of drugs allows the use of lower concentrations of each compound. This fact is of great importance, as one problem with using high doses of antioxidants is that they can become toxic. Studies have reported that ebselen at high doses impaired glutamate transmission as part of its toxic effects, and low doses prevent glutamate-mediated neuronal death associated with its antioxidant and/or antiinflammatory properties (Nogueira et al., 2003; Porciuncula et al., 2001, 2003). In the same manner, diphenyl diselenide has been demonstrated to exhibit neuroprotective, antiinflammatory, and antinociceptive activity (Meotti et al., 2004; Nogueira et al., 2003, 2004), although exposure to high doses of diphenyl diselenide induces seizure and death in rat pups and alters glutamatergic homeostasis (uptake and binding) *in vitro* and *in vivo* (Nogueira et al., 2001; Prigol et al., 2007). Thus, the combination of therapies might decrease the dosages for each agent and consequently reduce the occurrence of adverse effects (Margaill et al., 2005).

In view of this, the main objective of this study was to investigate whether the combined use of guanosine and organoselenium compounds could be more effective against glutamate-induced oxidative stress than the individual compounds. Indeed, the combination of guanosine and organoselenium compounds prevented glutamate-triggered ROS production in striatal, cortical, and hippocampal slices. These results corroborate the idea that the combined treatment is more effective than the isolated use of each compound. Additionally, a recent study demonstrated that organoselenium compounds used in combination with guanosine can protect rat brain cortical slices against methylmercury-induced ROS production related to glutamatergic system disturbances (Roos et al., 2009).

Another point that should be noted is the different responses of the brain regions to glutamate, guanosine, and organoselenium compounds. According to our results, cortical and hippocampal slices were more sensitive to the deleterious effects of glutamate than striatal slices were, as observed by the ROS and TBARS production induced by 10 mM glutamate. In addition, the inhibition of glutamate uptake was more pronounced in cortical and hippocampal slices. One explanation for these differences could be the different concentrations and distribution of glutamate receptors and transporters among the brain regions (Erecinska and Silver, 1996; Maiti et al., 2006). Glutamatergic synapses are more abundant in the cortex and hippocampus than in the striatum (Lehre et al., 1995). These

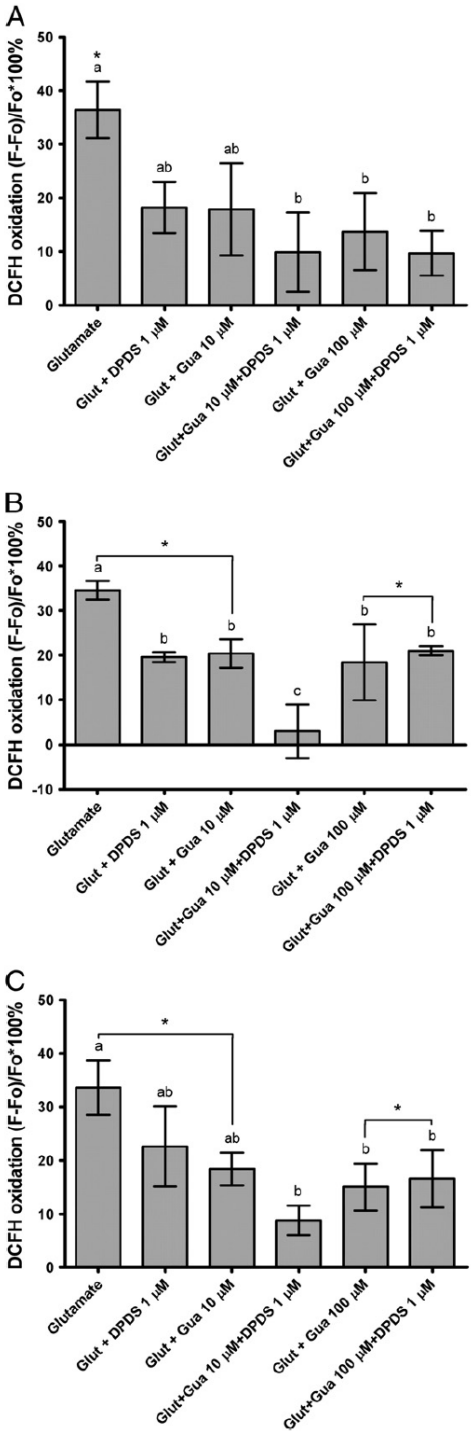
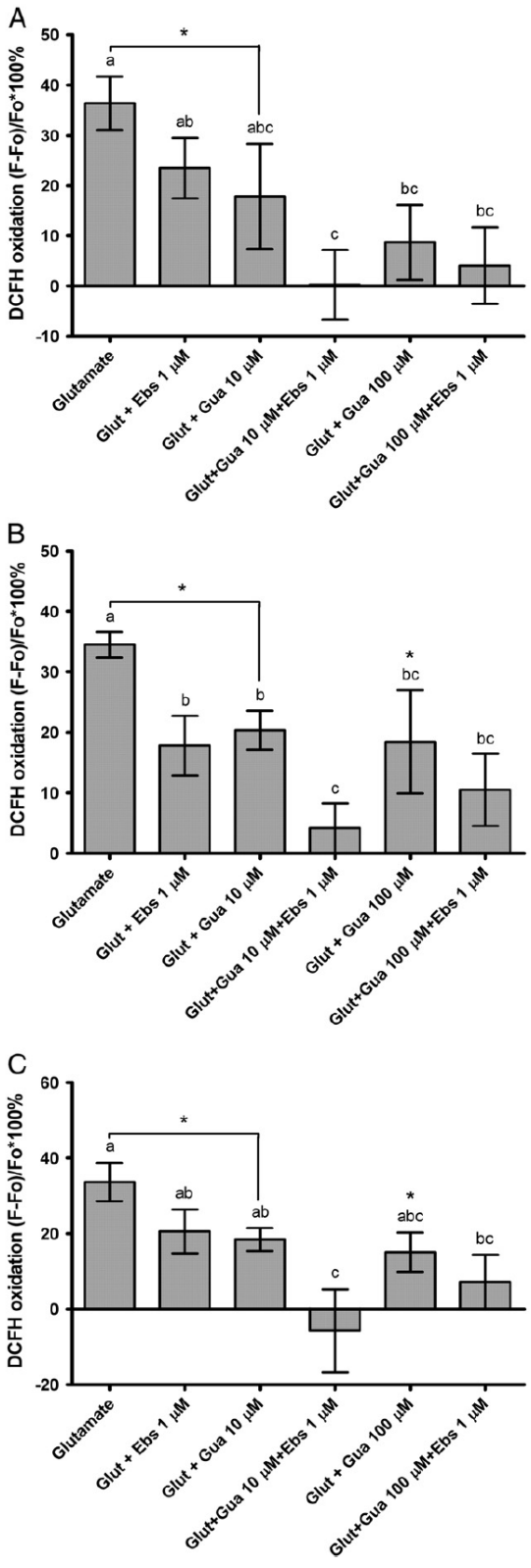



**Fig. 5 – Effect of the combination of guanosine (Gua) and diphenyl diselenide (DPDS) on glutamate (Glut)-induced ROS production in striatal (A), cortical (B), and hippocampal (C) slices from rats. Data are expressed as the mean ± S.E.M., n = 6. Experiments were performed in duplicate. Bars with different letters are significantly different ($p < 0.05$) from each other. Asterisk indicates significant differences from the control ($p < 0.05$).**

Please cite this article as: Dalla Corte, C.L., et al., The combination of organoselenium compounds and guanosine prevents glutamate-induced oxidative stress in different regions of rat brains, Brain Res. (2011), doi:10.1016/j.brainres.2011.10.049




findings could explain the lower susceptibility of striatal slices to glutamate compared to cortical and hippocampal slices.

Moreover, cortical and hippocampal slices were more sensitive to guanosine than striatal slices regarding glutamate-induced ROS formation, which could also be explained by the higher glutamatergic activity of these regions. However, the effects of guanosine on glutamate uptake did not differ among the brain regions. Therefore, other mechanisms could also be involved in the ability of guanosine to decrease ROS production. In addition to the stimulation of glutamate uptake, the decrease in glutamate binding and the scavenging of hydroxyl radicals induced by guanosine may also contribute to the protection and to the different sensitivity observed among the brain regions (Gudkov et al., 2006; Rubin et al., 1997).

The response of each region to the effect of the organoselenium compounds on ROS production varied according to the concentrations used. The concentrations of organoselenium compounds necessary to counteract ROS production were lower in striatal and cortical slices than in the hippocampal slices. In addition, the combined treatments were less effective in the striatal slices than in hippocampal and cortical slices, which could also be a consequence of the differences in the glutamatergic activity among these brain regions. Although we attribute the neuroprotection afforded by the organoselenium compounds to its antioxidant properties, we cannot rule out the involvement of other mechanisms.

The discovery and development of potent antioxidant agents has been one of the most promising approaches in the search for treatments for CNS injury (Gilgun-Sherki et al., 2002). Although some of the antioxidants exhibited efficacy in animal models, most of them did not exhibit beneficial effects in clinical trials performed to date (Gilgun-Sherki et al., 2002). The combination of antioxidants and substances capable of modulating glutamatergic activity would be more effective than the compounds alone because this combination could diminish oxidative stress generated via overactivation of NMDA receptors and still could diminish excess glutamate in synaptic clefts (Salom et al., 2004). Our findings supported this idea and demonstrated that organoselenium compounds in combination with guanosine are promising tools against glutamate-induced oxidative injury in CNS disorders. Furthermore, additional studies are necessary to determine the efficacy of the combination of organoselenium compounds with guanosine in reducing the prooxidative effects of glutamate in an *in vivo* model.

## 4. Experimental procedure

### 4.1. *Materials*

[$^3$H]-glutamic acid was purchased from Amersham Biosciences (Amersham, Bucks, UK). Guanosine, TBA, and MTT were

**Fig. 6 – Effect of the combination of guanosine (Gua) and ebselen (Ebs) on glutamate (Glut)-induced ROS production in striatal (A), cortical (B), and hippocampal (C) slices from rats. Data are expressed as the mean ± S.E.M., n = 6. Experiments were performed in duplicate. Bars with different letters are significantly different ($p < 0.05$) from each other. Asterisk indicates significant differences from the control ($p < 0.05$).**

Please cite this article as: Dalla Corte, C.L., et al., The combination of organoselenium compounds and guanosine prevents glutamate-induced oxidative stress in different regions of rat brains, Brain Res. (2011), doi:10.1016/j.brainres.2011.10.049

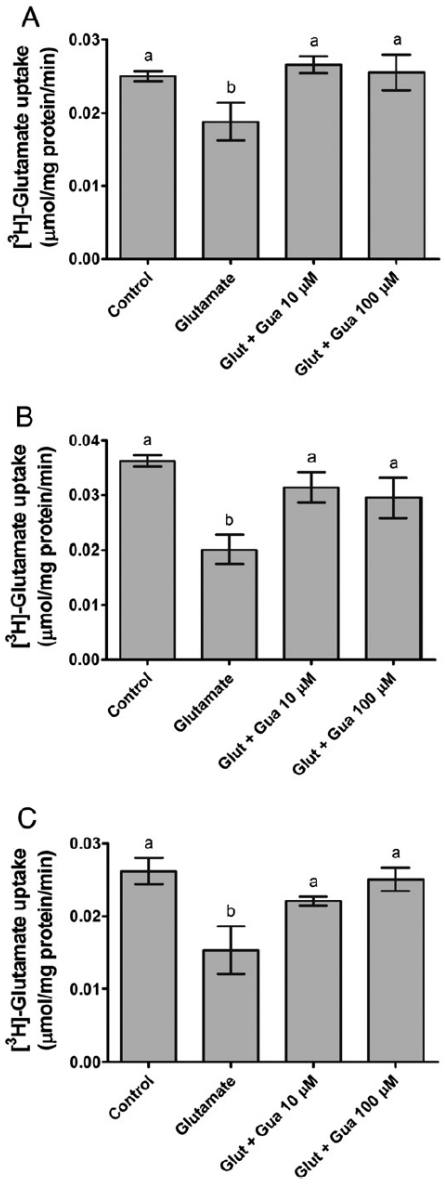



**Fig. 7 – Effect of guanosine (Gua) and glutamate (Glut) on [$^3$H]-glutamate uptake in striatal (A), cortical (B), and hippocampal (C) slices from rats. Data are expressed as the mean ± S.E.M., n = 4. Experiments were performed in duplicate. Bars with different letters are significantly different ($p < 0.05$) from each other.**

obtained from Sigma Aldrich (St Louis, MO, USA). DCFH was purchased from Molecular Probes (Eugene, OR, USA). Ebselen was synthesized with the method described by Engman and Hallberg (1989). Diphenyl diselenide was synthesized with the method described by Paulmier (1986). Solutions of organochalcogens were prepared freshly in dimethylsulfoxide (DMSO), and the final concentration of DMSO in the experiments was 2%. All other chemicals were of analytical grade and purchased from local commercial suppliers.

### 4.2. *Animals*

Male adult Wistar rats (250–350 g) from our own breeding colony were kept in cages of 4 animals each. They were placed in a room with controlled temperature (22±3 °C) on a 12-h light/dark cycle with lights on at 7:00 a.m. and continuous access to food and water. Our institutional protocols on animal experimentation, which were designed to minimize suffering and limit the number of animals sacrificed, were followed throughout the study. All experiments were conducted in accordance with the Committee on Care and Use of Experimental Animal Resources of the Federal University of Santa Maria, Brazil.

### 4.3. *Slice preparation*

Animals were sacrificed by decapitation. The brain was quickly removed, placed on ice, and then dissected into 3 specific regions: the cortex, the hippocampus, and the striatum. Slices (0.4-mm thick) of the brain regions were rapidly prepared using a McIlwain Tissue Chopper and then placed in ice-cold artificial cerebrospinal fluid (ACSF) containing 120 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 1.5 mM $CaCl_2$, 1.25 mM $NaH_2PO_4$, 35 mM $NaHCO_3$, and 10 mM D-glucose. The buffer was bubbled with 95% $O_2$ and 5% $CO_2$ up to pH 7.4 throughout the experiments.

### 4.4. *Lipid peroxidation assay*

Lipid peroxidation in the cortex, hippocampus, and striatum was evaluated by the TBARS method according to Rios and Santamaria (1991). The slices (5) were preincubated with L-glutamate (0.01, 0.1, 1, and 10 mM) for 2 h in oxygenated ACSF. For the TBARS assay, the slices were homogenized in 500 µL of ice-cold ACSF, and 1 mL of the TBA reagent (15% trichloroacetic acid, 0.375% TBA, and 2.5% v/v HCl) was added. After 30 min of incubation, samples were centrifuged (3000×*g*, 15 min), and the TBARS levels were measured at 532 nm in the supernatant. The final amounts of TBARS, mostly MDA, were calculated by interpolation of values in an MDA standard curve and corrected by the content of protein per sample. The results were calculated as nmol of TBARS/mg of protein and expressed as percent of controls.

### 4.5. *Cellular viability assay*

Cellular viability in the cortex, hippocampus, and striatum was quantified by measuring the reduction of MTT to a dark violet formazan product by mitochondrial dehydrogenases (Mosmann, 1983). The slices (2) were incubated with L-glutamate (0.01, 0.1, 1, and 10 mM) for 2 h in oxygenated ACSF. After incubation, the slices were washed twice with 1 mL of ACSF. The MTT reduction assays were performed in plates containing 1 mL of ACSF, and the reaction was started by adding 0.5 mg/mL MTT. After 1 h of incubation at 37 °C, the medium was removed, and the precipitated formazan in the slices was solubilized in DMSO. The rate of MTT reduction was measured spectrophotometrically at 570 nm. The results

Please cite this article as: Dalla Corte, C.L., et al., The combination of organoselenium compounds and guanosine prevents glutamate-induced oxidative stress in different regions of rat brains, Brain Res. (2011), doi:10.1016/j.brainres.2011.10.049

were expressed as percent of MTT reduction with respect to control values.

### 4.6. LDH release assay

LDH release, an index of cell disruption, was assessed in slices of the cortex, hippocampus, and striatum. The slices (2) were incubated with L-glutamate (0.01, 0.1, 1, and 10 mM) by 2 h in oxygenated ACSF. After this period, the total incubation medium of the slices (1 mL) was removed and mixed with reaction medium containing 100 mM glycine–KOH buffer (pH 10), 50 mM lactate, and 1 mM $NAD^+$. The enzyme activity was measured by determining the amount of NADH formed at room temperature at 340 nm (Pereira et al., 1991). Total LDH activity was determined by the same procedure described above after adding a final concentration of 10% Triton X-100 and disrupting the slice by homogenization. LDH leakage was estimated as the ratio between the LDH activity in the incubation medium and that of the whole slice content. The results were expressed as percent of LDH activity with respect to control values.

### 4.7. Estimation of ROS generation

Generation of ROS was estimated with the fluorescent probe DCFH by the method described by Ali et al. (1992). Slices (2) of the cortex, hippocampus, and striatum were preincubated with 100 μM guanosine in oxygenated ACSF for 15 min at 37 °C. Then, L-glutamate (0.01, 0.1, 1, and 10 mM) was added to the slices and incubated for another 2 h in order to create a dose–response curve of DCFH oxidation. The glutamate concentration of 1 mM was used to perform the other experiments with guanosine and organoselenium compounds because it was the lowest concentration of glutamate that significantly induced ROS generation (DCFH oxidation), which is supported by other studies (Kannurpatti, et al., 2004; Molz, et al., 2011). Then, we performed dose–response curve of DCFH oxidation with L-glutamate (1 mM) *versus* guanosine (0.5–200 μM), diphenyl diselenide (0.05–100 μM), and ebselen (0.01–100 μM). To study the effect of the combination of guanosine and organoselenium compounds, the concentrations of 10 and 100 μM were chosen for guanosine, and 1 μM was chosen for diphenyl diselenide and ebselen. For the DCFH oxidation assay, the slices were homogenized in 2.5 mL of saline solution (0.9% NaCl). Aliquots of 2.5 mL were incubated in the presence of DCFH (5 μM) at 37 °C for 1 h. Fluorescence was measured using excitation and emission wavelengths of 488 and 522 nm, respectively. A calibration curve was established with standard DCF. ROS levels were expressed as percent of control as follows: (F – Fo)/Fo*100, where F is the fluorescence intensity of the control slices and Fo is the fluorescence intensity of the slices treated with glutamate, guanosine, and/or organoselenium compounds. The data with organoselenium compounds alone was omitted because they did not exert any effect *per se* on the fluorescence measurements (DCFH fluorescence).

### 4.8. [$^3$H]-glutamate uptake assay

For the glutamate uptake measurement, slices (2) of the cortex, hippocampus, and striatum were preincubated with and without 10 and 100 μM guanosine in oxygenated ACSF during 15 min at 37 °C. Then, 1 mM L-glutamate was added to the slices and incubated for another 2 h. At the end of the incubation period, the slices were washed twice with ACSF and adjusted to pH 7.2. Glutamate uptake was performed according to Frizzo et al. (2002) with some modifications. Briefly, uptake was performed at 35 °C by adding 100 μM unlabeled L-glutamate and 1 μM (0.33 μCi/mL) [$^3$H]-glutamate. The reaction was stopped after 7 (cortex), 5 (hippocampus), or 3 (striatum) min by washing twice with 1 mL of cold ACSF, immediately followed by the addition of 0.5 N NaOH, which was kept overnight. Sodium-independent uptake was determined using choline chloride instead of sodium chloride, which was subtracted from the total uptake to obtain the sodium-dependent uptake. Incorporated radioactivity was determined with a Packard scintillator (TRI CARB 2100 TR—PerkinElmer, Waltham, MA, USA).

### 4.9. Protein measurement

Protein concentrations were assayed by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

### 4.10. Statistical analysis

Data were analyzed statistically by 1-way ANOVA, followed by Duncan's post-hoc tests. The results were considered statistically significant when $p<0.05$.

## Acknowledgments

Supported by FAPERGS (Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), FINEP (Rede Instituto Brasileiro de Neurociência (IBN-Net) # 01.06.0842-00) and INCT for Excitotoxicity and Neuroprotection — MCT/CNPq. C.L. Dalla Corte, F. Dobrachinski, J.B.T. Rocha and F.A.A. Soares are the recipients of CNPq fellowships.

## REFERENCES

Ali, S.F., LeBel, C.P., Bondy, S.C., 1992. Reactive oxygen species formation as a biomarker of methylmercury and trimethyltin neurotoxicity. Neurotoxicology 13, 637–648.

Berr, C., Balansard, B., Arnaud, J., Roussel, A.M., Alperovitch, A., 2000. Cognitive decline is associated with systemic oxidative stress: the EVA study. Etude du Vieillissement Arteriel. J. Am. Geriatr. Soc. 48, 1285–1291.

Burger, M., Fachinetto, R., Calegari, L., Paixao, M.W., Braga, A.L., Rocha, J.B., 2004. Effects of age on reserpine-induced orofacial dyskinesia and possible protection of diphenyl diselenide. Brain Res. Bull. 64, 339–345.

Burger, M.E., Fachinetto, R., Wagner, C., Perottoni, J., Pereira, R.P., Zeni, G., Rocha, J.B., 2006. Effects of diphenyl-diselenide on orofacial dyskinesia model in rats. Brain Res. Bull. 70, 165–170.

Centuriao, F.B., Corte, C.L., Paixao, M.W., Braga, A.L., Zeni, G., Emanuelli, T., Rocha, J.B., 2005. Effect of ebselen and organochalcogenides on excitotoxicity induced by glutamate in isolated chick retina. Brain Res. 1039, 146–152.

Choi, D.W., 1992. Excitotoxic cell death. J. Neurobiol. 23, 1261–1276.

Ciccarelli, R., Ballerini, P., Sabatino, G., Rathbone, M.P., D'Onofrio, M., Caciagli, F., Di Iorio, P., 2001. Involvement of astrocytes in purine-mediated reparative processes in the brain. Int. J. Dev. Neurosci. 19, 395–414.

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

Danbolt, N.C., 2001. Glutamate uptake. Prog. Neurobiol. 65, 1–105.

Engman, L., Hallberg, A., 1989. Expedient synthesis of ebselen and related-compounds. J. Org. Chem. 54, 2964–2966.

Engman, L., Cotgreave, I., Angulo, M., Taylor, C.W., Paine-Murrieta, G.D., Powis, G., 1997. Diaryl chalcogenides as selective inhibitors of thioredoxin reductase and potential antitumor agents. Anticancer Res. 17, 4599–4605.

Erecinska, M., Silver, I.A., 1996. Calcium handling by hippocampal neurons under physiologic and pathologic conditions. Adv. Neurol. 71, 119–136.

Frizzo, M.E., Lara, D.R., Dahm, K.C., Prokopiuk, A.S., Swanson, R.A., Souza, D.O., 2001. Activation of glutamate uptake by guanosine in primary astrocyte cultures. Neuroreport 12, 879–881.

Frizzo, M.E., Lara, D.R., Prokopiuk Ade, S., Vargas, C.R., Salbego, C.G., Wajner, M., Souza, D.O., 2002. Guanosine enhances glutamate uptake in brain cortical slices at normal and excitotoxic conditions. Cell. Mol. Neurobiol. 22, 353–363.

Frizzo, M.E., Antunes Soares, F.A., Dall'Onder, L.P., Lara, D.R., Swanson, R.A., Souza, D.O., 2003. Extracellular conversion of guanine-based purines to guanosine specifically enhances astrocyte glutamate uptake. Brain Res. 972, 84–89.

Funchal, C., Moretto, M.B., Vivian, L., Zeni, G., Rocha, J.B., Pessoa-Pureur, R., 2006. Diphenyl ditelluride- and methylmercury-induced hyperphosphorilation of the high molecular weight neurofilament subunit is prevented by organoselenium compounds in cerebral cortex of young rats. Toxicology 222, 143–153.

Ghisleni, G., Porciuncula, L.O., Cimarosti, H., Batista, T.R.J., Salbego, C.G., Souza, D.O., 2003. Diphenyl diselenide protects rat hippocampal slices submitted to oxygen–glucose deprivation and diminishes inducible nitric oxide synthase immunocontent. Brain Res. 986, 196–199.

Ghisleni, G., Porciuncula, L.O., Mioranzza, S., Boeck, C.R., Rocha, J.B., Souza, D.O., 2008. Selenium compounds counteract the stimulation of ecto-nucleotidase activities in rat cultured cerebellar granule cells: putative correlation with neuroprotective effects. Brain Res. 1221, 134–140.

Gilgun-Sherki, Y., Rosenbaum, Z., Melamed, E., Offen, D., 2002. Antioxidant therapy in acute central nervous system injury: current state. Pharmacol. Rev. 54, 271–284.

Gudkov, S.V., Shtarkman, I.N., Smirnova, V.S., Chernikov, A.V., Bruskov, V.I., 2006. Guanosine and inosine display antioxidant activity, protect DNA in vitro from oxidative damage induced by reactive oxygen species, and serve as radioprotectors in mice. Radiat. Res. 165, 538–545.

Halliwell, B., 2006. Oxidative stress and neurodegeneration: where are we now? J. Neurochem. 97, 1634–1658.

Janero, D.R., 1990. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radic. Biol. Med. 9, 515–540.

Kade, I.J., Nogueira, C.W., Rocha, J.B., 2009. Diphenyl diselenide and streptozotocin did not alter cerebral glutamatergic and cholinergic systems but modulate antioxidant status and sodium pump in diabetic rats. Brain Res. 1284, 202–211.

Kannurpatti, S.S., Sanganahalli, B.G., Mishra, S., Joshi, P.G., Joshi, N.B., 2004. Glutamate-induced differential mitochondrial response in young and adult rats. Neurochem. Int. 44, 361–369.

Lehre, K.P., Levy, L.M., Ottersen, O.P., Storm-Mathisen, J., Danbolt, N.C., 1995. Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. J. Neurosci. 15, 1835–1853.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Mailly, F., Marin, P., Israel, M., Glowinski, J., Premont, J., 1999. Increase in external glutamate and NMDA receptor activation contribute to $H_2O_2$-induced neuronal apoptosis. J. Neurochem. 73, 1181–1188.

Maiti, P., Singh, S.B., Sharma, A.K., Muthuraju, S., Banerjee, P.K., Ilavazhagan, G., 2006. Hypobaric hypoxia induces oxidative stress in rat brain. Neurochem. Int. 49, 709–716.

Margaill, I., Plotkine, M., Lerouet, D., 2005. Antioxidant strategies in the treatment of stroke. Free Radic. Biol. Med. 39, 429–443.

McEntee, W.J., Crook, T.H., 1993. Glutamate: its role in learning, memory, and the aging brain. Psychopharmacology (Berl) 111, 391–401.

Mejia-Toiber, J., Montiel, T., Massieu, L., 2006. D-beta-hydroxybutyrate prevents glutamate-mediated lipoperoxidation and neuronal damage elicited during glycolysis inhibition in vivo. Neurochem. Res. 31, 1399–1408.

Meotti, F.C., Borges, V.C., Zeni, G., Rocha, J.B., 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., Rocha, J.B., 2004. Protective role of aryl and alkyl diselenides on lipid peroxidation. Environ. Res. 94, 276–282.

Molz, S., Dal-Cim, T., Budni, J., Martín-de-Saavedra, M.D., Egea, J., Romero, A., del Barrio, L., Rodrigues, A.L., López, M.G., Tasca, C.I., 2011. Neuroprotective effect of guanosine against glutamate-induced cell death in rat hippocampal slices is mediated by the phosphatidylinositol-3 kinase/akt/glycogen synthase kinase 3b pathway activation and inducible nitric oxide synthase inhibition. J. Neurosci. Res. 89, 1400–1408.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Mugesh, G., du Mont, W.W., Sies, H., 2001. Chemistry of biologically important synthetic organoselenium compounds. Chem. Rev. 101, 2125–2179.

Namura, S., Nagata, I., Takami, S., Masayasu, H., Kikuchi, H., 2001. Ebselen reduces cytochrome c release from mitochondria and subsequent DNA fragmentation after transient focal cerebral ischemia in mice. Stroke 32, 1906–1911.

Nogueira, C.W., Rotta, L.N., Perry, M.L., Souza, D.O., da Rocha, J.B., 2001. Diphenyl diselenide and diphenyl ditelluride affect the rat glutamatergic system in vitro and in vivo. Brain Res. 906, 157–163.

Nogueira, C.W., Quinhones, E.B., Jung, E.A., Zeni, G., Rocha, J.B., 2003. Anti-inflammatory and antinociceptive activity of diphenyl diselenide. Inflamm. Res. 52, 56–63.

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

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

Paulmier, C., 1986. Selenoorganic functional groups. In: Paulmier, C. (Ed.), Selenium Reagents and Intermediates in Organic Synthesis. Pergamon Press, Oxford, pp. 25–51.

Pellegrini-Giampietro, D.E., Cherici, G., Alesiani, M., Carla, V., Moroni, F., 1990. Excitatory amino acid release and free radical formation may cooperate in the genesis of ischemia-induced neuronal damage. J. Neurosci. 10, 1035–1041.

Pereira, M.E., Bordignon, A.M., Burger, C., Huang, C.I., Rocha, J.B., 1991. Long-term treatment with 2,5-hexanedione has no effect on the specific activity of some brain and liver glycolytic enzymes of adult rats. Braz. J. Med. Biol. Res. 24, 735–740.

Porciuncula, L.O., Rocha, J.B., Boeck, C.R., Vendite, D., Souza, D.O., 2001. Ebselen prevents excitotoxicity provoked by glutamate in rat cerebellar granule neurons. Neurosci. Lett. 299, 217–220.

Please cite this article as: Dalla Corte, C.L., et al., The combination of organoselenium compounds and guanosine prevents glutamate-induced oxidative stress in different regions of rat brains, Brain Res. (2011), doi:10.1016/j.brainres.2011.10.049

Porciuncula, L.O., Rocha, J.B., Cimarosti, H., Vinade, L., Ghisleni, G., Salbego, C.G., Souza, D.O., 2003. Neuroprotective effect of ebselen on rat hippocampal slices submitted to oxygen–glucose deprivation: correlation with immunocontent of inducible nitric oxide synthase. Neurosci. Lett. 346, 101–104.

Posser, T., Franco, J.L., dos Santos, D.A., Rigon, A.P., Farina, M., Dafre, A.L., Rocha, J.B.T., Leal, R.B., 2008. Diphenyl diselenide confers neuroprotection against hydrogen peroxide toxicity in hippocampal slices. Brain Res. 1199, 138–147.

Prigol, M., Wilhelm, E.A., Schneider, C.C., Rocha, J.B., Nogueira, C.W., Zeni, G., 2007. Involvement of oxidative stress in seizures induced by diphenyl diselenide in rat pups. Brain Res. 1147, 226–232.

Rathbone, M.P., Middlemiss, P.J., Gysbers, J.W., Andrew, C., Herman, M.A., Reed, J.K., Ciccarelli, R., Di Iorio, P., Caciagli, F., 1999. Trophic effects of purines in neurons and glial cells. Prog. Neurobiol. 59, 663–690.

Rios, C., Santamaria, A., 1991. Quinolinic acid is a potent lipid peroxidant in rat brain homogenates. Neurochem. Res. 16, 1139–1143.

Roos, D.H., Puntel, R.L., Santos, M.M., Souza, D.O., Farina, M., Nogueira, C.W., Aschner, M., Burger, M.E., Barbosa, N.B., Rocha, J.B., 2009. Guanosine and synthetic organoselenium compounds modulate methylmercury-induced oxidative stress in rat brain cortical slices: involvement of oxidative stress and glutamatergic system. Toxicol. In Vitro 23, 302–307.

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

Rubin, M.A., Medeiros, A.C., Rocha, P.C., Livi, C.B., Ramirez, G., Souza, D.O., 1997. Effect of guanine nucleotides on [$^3$H] glutamate binding and on adenylate cyclase activity in rat brain membranes. Neurochem. Res. 22, 181–187.

Salom, J.B., Perez-Asensio, F.J., Burguete, M.C., Marin, N., Pitarch, C., Torregrosa, G., Romero, F.J., Alborch, E., 2004. Single-dose ebselen does not afford sustained neuroprotection to rats subjected to severe focal cerebral ischemia. Eur. J. Pharmacol. 495, 55–62.

Schmidt, A.P., Avila, T.T., Souza, D.O., 2005. Intracerebroventricular guanine-based purines protect against seizures induced by quinolinic acid in mice. Neurochem. Res. 30, 69–73.

Soares, F.A., Schmidt, A.P., Farina, M., Frizzo, M.E., Tavares, R.G., Portela, L.V., Lara, D.R., Souza, D.O., 2004. Anticonvulsant effect of GMP depends on its conversion to guanosine. Brain Res. 1005, 182–186.

Stangherlin, E.C., Luchese, C., Pinton, S., Rocha, J.B., Nogueira, C.W., 2008. Sub-chronical exposure to diphenyl diselenide enhances acquisition and retention of spatial memory in rats. Brain Res. 1201, 106–113.

Takasago, T., Peters, E.E., Graham, D.I., Masayasu, H., Macrae, I.M., 1997. Neuroprotective efficacy of ebselen, an anti-oxidant with anti-inflammatory actions, in a rodent model of permanent middle cerebral artery occlusion. Br. J. Pharmacol. 122, 1251–1256.

Trotti, D., Danbolt, N.C., Volterra, A., 1998. Glutamate transporters are oxidant-vulnerable: a molecular link between oxidative and excitotoxic neurodegeneration? Trends Pharmacol. Sci. 19, 328–334.

Walton, H.S., Dodd, P.R., 2007. Glutamate–glutamine cycling in Alzheimer's disease. Neurochem. Int. 50, 1052–1066.

Warren, I., 2002. Facilitating infant adaptation: the nursery environment. Semin. Neonatol. 7, 459–467.

Wilson, S.R., Zucker, P.A., Huang, R.R.C., Spector, A., 1989. Development of synthetic compounds with glutathione-peroxidase activity. J. Am. Chem. Soc. 111, 5936–5939.

Yamaguchi, T., Sano, K., Takakura, K., Saito, I., Shinohara, Y., Asano, T., Yasuhara, H., 1998. Ebselen in acute ischemic stroke: a placebo-controlled, double-blind clinical trial. Ebselen Study Group. Stroke 29, 12–17.

Zhao, R., Holmgren, A., 2002. A novel antioxidant mechanism of ebselen involving ebselen diselenide, a substrate of mammalian thioredoxin and thioredoxin reductase. J. Biol. Chem. 277, 39456–39462.

Please cite this article as: Dalla Corte, C.L., et al., The combination of organoselenium compounds and guanosine prevents glutamate-induced oxidative stress in different regions of rat brains, Brain Res. (2011), doi:10.1016/j.brainres.2011.10.049

## 2.2 – O DISSELENETO DE DIFENILA PREVINE A DISFUNÇÃO MITOCONDRIAL INDUZIDA POR METILMERCÚRIO EM FATIAS DE FÍGADO DE RATOS

**Manuscrito 1**

## DIPHENYL DISELENIDE PREVENTS METHYLMERCURY-INDUCED MITOCHONDRIAL DYSFUNCTION IN RAT LIVER SLICES

CRISTIANE L. DALLA CORTE, MICHAEL ASCHNER, JOÃO B. T. ROCHA

**Diphenyl diselenide prevents methylmercury-induced mitochondrial dysfunction in rat liver slices.**

Cristiane L. Dalla Corte[a,*], Michael Aschner[b,c], João B. T. Rocha[a,*]

[a]*Departamento de Química, Programa de Pós-graduação em Ciências Biológicas: Bioquímica Toxicológica, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Cep 97105-900, Santa Maria, RS, Brazil.*

[b]*Department of Pharmacology, Vanderbilt University Medical Center, Nashville, TN 37232, USA.*

[c]*Department of Pediatrics, Vanderbilt University Medical Center, Nashville, TN 37232, USA.*

**Correspondence to:*
*Cristiane Lenz Dalla Corte and João Batista Teixeira Rocha*
*Universidade Federal de Santa Maria – Dep. de Química*
*Cep 97105-900, Santa Maria, RS, Brasil.*
*Tel: #55-55-3220-8140*
*Fax: #55-55-3220-8978*
E-mail adresses: jbtrocha@yahoo.com.br (J.B.T. Rocha), crisbioq@yahoo.com.br (C.L. Dalla Corte).

**Abstract**

Methylmercury (MeHg) is an environmental contaminant with toxic effects in multiple organs. The present study was designed to investigate the possible protective effects of diphenyl diselenide (DPDS), a seleno-organic compound, against MeHg-induced mitochondrial dysfunction in rat liver slices. The liver slices were treated with MeHg (25 μM) and/ or DPDS (0.5, 1, 5 μM) for 30 min at 37°C, then mitochondria was isolated from these slices, and the reactive oxygen species (ROS) formation, oxygen consumption, membrane potential (ΔΨm) and mitochondrial function were assessed. MeHg decreased the mitochondrial metabolic function, increased ROS production, impaired oxygen consumption and collapsed the ΔΨm. DPDS protected against the MeHg-induced ROS generation and prevented the decrease in the respiratory rate and in the mitochondrial metabolic function [measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction]. DPDS (0.5 μM) blunted the MeHg-induced ΔΨm collapse; however at 5 μM, DPDS alone decreased ΔΨm, yet partially protected the mitochondria from MeHg-induced depolarization. The protection afforded by DPDS against the MeHg-induced mitochondrial dysfunction may be associated with DPDS's metabolism to a selenol intermediate, forming inert complex(es) with MeHg, thus effectively decreasing its toxicity. Furthermore, the selenol intermediate of DPDS (selenophenol) may have direct antioxidant properties. In conclusion, the results demonstrate that low DPDS concentrations effectively prevent the MeHg-induced mitochondrial dysfunction.

**Keywords:** Methylmercury; seleno-organic compounds; ROS production; mitochondrial permeability transition pore; mitochondrial dysfunction.

**Introduction**

Methylmercury (MeHg) is a highly toxic environmental contaminant with worldwide public health concern (Clarkson et al., 2003). MeHg is generated by biomethylation of inorganic Hg in aquatic sediments, leading to its subsequent bioaccumulation in the aquatic food chain (Clarkson et al., 2003). Accordingly, the consumption of contaminated fish and sea food is considered the main source of human exposure to MeHg (Clarkson et al., 2003; Dorea et al., 2006; Lando and Zhang, 2011).

The high affinity of MeHg for sulfhydryl (-SH) groups is considered an important mechanism in mediating MeHg toxicity since the binding of MeHg to –SH groups inactivates enzymes and disrupts biochemical and signaling pathways (Farina et al., 2011a,b; Valko et al., 2005). ROS formation has also been implicated in MeHg toxicity, reflecting the interaction of MeHg with thiol- and selenol-containing proteins (Aschner et al., 2007; Ceccatelli et al., 2010; Farina et al., 2009, 2011a,b). ROS can cause oxidative damage to mitochondria, compromising mitochondrial function, thus further perpetuating ROS production (Halliwell and Gutteridge, 1999). MeHg can also induce the opening of the mitochondrial permeability transition pore (MTP), a process activated by excess of $Ca^{2+}$ within the mitochondria (Limke et al., 2003, 2004; Limke and Atchison, 2002). Opening of the MTP allows for the passive diffusion of molecules <1.5 kDa, leading to a collapse of the mitochondrial inner membrane potential ($\Delta\Psi m$) (Limke et al., 2004). Loss of the $\Delta\Psi m$ can result in mitochondrial swelling, movement of metabolites across the inner membrane, defective oxidative phosphorylation, cessation of ATP synthesis, generation of ROS and ultimately cell death (Atchison and Hare, 1994).

Selenium is an essential trace element for mammals and a major component of antioxidant seleno-enzymes, such as glutathione peroxidase (GPx) and thioredoxin reductase (TrxR) (Allan et al., 1999; Flohe, 1988; Holmgren, 1989). The discovery that selenium has a fundamental role in the antioxidant activity of these enzymes has led to great interest in organic selenium compounds, as potential remedies in combating oxidative stress in biological systems (Nogueira and Rocha 2011, 2010).

Both organic and inorganic selenium can influence the deposition of MeHg in the body and protect against its toxicity in animals (Choi et al., 2008; Fredriksson et al., 1993; Ganther, 1971; Glaser et al., 2010). Mechanism(s) of selenium protection against MeHg-induced toxicity may be related either to the formation of inert complexes between inorganic or organic selenium compounds and MeHg, or by the formation of readily excretable MeHg complexes (de Freitas et al., 2009; Pinheiro et al., 2009).

The potential applications of antioxidants in the treatment of disease related to oxidative stress have stimulated the synthesis and study, harnessing the beneficial biological properties of seleno-organic compounds with GPx-like activity (Mugesh et al., 2001; Nogueira et al., 2004; Parnham and Graf, 1991). Among these compounds, diphenyl diselenide (DPDS) was shown to have efficacious properties, protecting a brain, liver and kidney from the overproduction of free radicals in a variety of *in vitro* and *in vivo* experimental models (Burger et al., 2004, 2006; Funchal et al., 2006; Ghisleni et al., 2003; Moretto et al., 2005; Nogueira et al., 2004). For example, DPDS protected against toxic effects of MeHg in mice and this protection was attributable to its antioxidant properties and its ability to reduce Hg body burden via the formation of complex(es) between MeHg and selenol (PhSeH), generating the readily excretable PhSeHgMe complex (de Freitas et al., 2009). Recently, we have demonstrated that DPDS can also be a substrate to brain and liver thioredoxin reductase (de Freitas et al. 2010, 2011). Consequently, its antioxidant and protective effects can be linked to either its thiol-peroxidase-like activity (GPx-like activity) or to its reduction to selenophenol by TrxR (see scheme 1).

Mitochondria are critical target organelles of MeHg toxicity and are involved in both physiological and pathological ROS production. Accordingly, targeting compounds with antioxidant properties to the mitochondria offer innovative pharmacological modalities in protecting against ROS-induced mitochondrial dysfunction. Taking into account the antioxidant properties of selenium and its ability to affect MeHg deposition in tissues, seleno-organic compounds should be considered as potential therapeutic agents against the MeHg toxicity. Accordingly, the present study was designed to test the hypothesis that DPDS, a seleno-organic compound, will offer protection against *in vitro* MeHg induced-mitochondrial dysfunction.

## Materials and Methods

### *Chemicals*

Chemicals, including ethylene glycol-bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), glutamic acid, rhodamine 123 and 2',7'-Dichlorofluorescin diacetate ($H_2$-DCFDA) were obtained from Sigma Aldrich (St Louis, MO, USA). MeHg was obtained from Merck (Rio de Janeiro, RJ, Brazil). DPDS was synthesized

using the method described by Paulmier (1986). DPDS solutions were prepared freshly in dimethylsulfoxide (DMSO) with a final DMSO concentration in the experiments set at 2%; at this concentration DMSO had no effects on the tested parameters. All other chemicals were of analytical grade and purchased from local commercial suppliers.

*Animals*

Male adult Wistar rats (250–350 g) from our own breeding colony were kept in cages of four animals each. They were placed in a room with controlled temperature (22 ± 3°C) on a 12 h light/dark cycle with lights on at 7:00 a.m., and had continuous access to *ad lib* food and water. Our institutional protocols for experiments with animals, designed to minimize suffering and limit the number of animals sacrificed, were followed throughout. All experiments were conducted in accordance with the Committee on Care and Use of Experimental Animal Resources of the Federal University of Santa Maria, Brazil.

*Slices Preparation*

Animals were sacrificed by decapitation. The liver was quickly removed, placed on ice, and slices (0.4 mm thick) were rapidly prepared using a Mcllwain Tissue Chopper and then placed in an ice-cold Krebs-Ringer solution containing: 120 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 1.5 mM $CaCl_2$, 1.25 mM $NaH_2PO_4$, 35 mM $NaHCO_3$ and 10 mM D-glucose. The buffer was bubbled with 95% $O_2$ and 5% $CO_2$ up to pH 7.4 throughout the experiments.

*Standard incubation procedure*

Slices of liver (60 slices) of rat were incubated for 30 min with/or without MeHg (25 µM) and/or DPDS (0.5, 1, 5 µM) at 37°C. Slices were then washed 3 times with a cold solution of 100 µM L-cysteine hydrochloride and homogenized for mitochondria isolation (see details below). The concentration of MeHg (25 µM) was based on a previous study (Roos et al., 2011).

*Isolation of rat liver mitochondria*

Rat liver mitochondria were isolated as previously described by Brustovetsky and Dubinsky (2000), with some modifications. The liver slices were rapidly weighed and homogenized in 1:5 (w/v) ice-cold 'isolation buffer I' containing 225 mM mannitol, 75 mM sucrose, 1 mM $K^+$-EGTA, 0.1% bovine serum albumin (BSA) and 10 mM $K^+$-HEPES, pH 7.2. Slices were then manually homogenized using a potter glass and centrifuged for 7 min at

2,000 g. The resulting supernatant was centrifuged for 10 min at 12,000 g and the first mitochondrial pellet was re-suspended in 'isolation buffer II' containing 225 mM mannitol, 75 mM sucrose, 1 mM $K^+$-EGTA, and 10 mM $K^+$-HEPES pH 7.2 and re-centrifuged at 12,000 g for 10 min. The supernatant was discarded and the final pellet was gently washed and resuspended in 'isolation buffer II' without EGTA.

*Measurements of mitochondrial transmembrane electrical potential (ΔΨm)*

Mitochondrial ΔΨm was estimated by fluorescence changes in rhodamine 123 (2 μM) recorded by RF-5301 Shimadzu spectrofluorometer (Kyoto, Japan) operating at excitation and emission wavelengths of 495 and 535 nm, with slit widths of 1.5 nm (Guo *et al.*, 1998). Mitochondria (1 mg protein) were added at 15 s of recording to 3 ml standard incubation buffer containing 100 mM sucrose, 65 mM KCl, 10 mM $K^+$-HEPES buffer (pH 7.2), 50 μM EGTA, 200 μM ADP, 400 μM $MgCl_2$, 1 mM Pi, 5 mM glutamate and 5 mM succinate. Responses were recorded for the ensuing 5 min. At 200 s of recording FCCP (1 μM) was added. Data on mitochondrial transmembrane electrical potential (ΔΨm) are presented as Arbitrary Fluorescence Units.

*Estimation of reactive oxygen species (ROS) production*

Mitochondrial ROS generation was determined spectrofluorimetrically, using the membrane permeable fluorescent dye $H_2$-DCFDA recorded by RF-5301 Shimadzu spectrofluorometer (Kyoto, Japan) operating at excitation and emission wavelengths of 488 and 525 nm, with slit widths of 3 nm (GarciaRuiz *et al.*, 1997). Mitochondria (0.3 mg protein) were added to 3 ml standard incubation buffer containing 100 mM sucrose, 65 mM KCl, 10 mM $K^+$-HEPES buffer (pH 7.2) and 50 μM EGTA. Responses were recorded for the ensuing 5 min. At 15 s of recording the $H_2$-DCFDA (1 μM) was added. When $CaCl_2$ (60 μM) was used it was added at 30 s of the recording. Data on ROS production are presented as Arbitrary Fluorescence Units.

*Assessment of mitochondrial metabolic function*

Mitochondrial metabolic function was assessed by the conversion of MTT to a dark violet formazan product by mitochondrial dehydrogenases (Mosmann, 1983). MTT (400 μg/ml) was added to the standard buffer (100 mM sucrose, 65 mM KCl, 10 mM $K^+$-HEPES buffer (pH 7.2), 50 μM EGTA, 200 μM ADP, 400 μM $MgCl_2$, 1 mM Pi, 5mM glutamate and 5 mM succinate) containing an aliquot of mitochondria (0.3 mg protein), and incubated for 45

min at $37^0$C. After 45 min the precipitated formazan was solubilized in DMSO. The rate of MTT reduction was measured spectrophotometrically at a wavelength of 570 nm. Results were expressed as the percentage of MTT reduction with respect to control values.

*Oxygen Uptake Measurements*

Oxygen uptake was measured using an oxymeter (Hansatech model with a Clark-type electrode) at 30 °C. The isolated rat liver mitochondria (1 mg protein) were incubated with 2 ml of the standard incubation buffer containing 100 mM sucrose, 65 mM KCl, 10 mM $K^+$-HEPES buffer (pH 7.2) and 50 µM EGTA. Succinate (5 mM) was placed in the medium in order to increase the respiratory state.

*Protein measurement*

Protein was assayed by the method of Lowry et al. (1951) with bovine serum albumin as standard.

*Statistical analysis*

Data were analyzed statistically by one-way analysis of variance (ANOVA), followed by Duncan's *post-hoc* tests. The results were considered statistically significant when $p<0.05$.

**Results**

*Effects of MeHg and DPDS on mitochondrial metabolic function*

Mitochondria isolated from liver slices previously treated with 25 µM MeHg reduced MTT less efficiently than mitochondria isolated from non-treated slices (Figure 1; $p<0.05$). Treatment of liver slices with DPDS did not alter the MTT reducing activity by isolated mitochondria. Notably, the co-treatment of liver slices with DPDS (0.5, 1, 5 µM) fully reversed the inhibition of MTT reduction caused by short-term MeHg treatment to levels indistinguishable from controls ($p<0.05$; Figure 1).

*Effects of MeHg and DPDS on ROS generation*

Oxidation of $H_2$-DCFH by mitochondria isolated from liver slices previously treated with 25 µM MeHg was significantly higher than that observed in mitochondria isolated from non-MeHg-treated slices (Figure 2; $p<0.05$). $CaCl_2$ (60 µM) increased ROS production ($H_2$-

DCFH oxidation) both in mitochondria isolated from MeHg-treated or control liver slices (Figure 2).

The co-treatment of slices with DPDS (0.5, 1, 5 µM) blunted mitochondrial MeHg-induced ROS generation ($p<0.05$, Fig. 3A and B). However, the protection was inversely related to the concentration (i.e., the highest concentration of DPDS tested afforded only a partial protection against MeHg induced-ROS generation, while the lowest concentration (0.5 µM) provided a complete protection). This may be related to the fact that 5 µM DPDS alone partially increased DCFH oxidation (Figure 3B).

*Effects of MeHg and DPDS on ΔΨm*

Polarization (ΔΨm) of mitochondria isolated from liver slices previously treated with 25 µM MeHg was significantly reduced vs. the polarization observed in mitochondria isolated from non-MeHg-treated slices (Figure 4; $p<0.05$). DPDS at 0.5 and 1 µM had no effect in mitochondrial ΔΨm when compared to mitochondria isolated from control liver slices; co-treatment of slices with 0.5 µM of DPDS blunted the pretreatment effect of MeHg on liver mitochondrial polarization (Figure 4A) whereas 0.1 µM of DPDS partially decreased the MeHg-induced mitochondrial depolarization (Figure 4B). In contrast, 5 µM DPDS caused a partial dissipation of mitochondrial ΔΨm and the co-treatment only partially decreased the MeHg-induced mitochondrial depolarization (Figure 4C).

*Effects of MeHg and DPDS on oxygen consumption*

Treatment of liver slices to MeHg significantly decreased the mitochondrial oxygen consumption as determined in the presence of succinate as a substrate ($p<0.005$, Table 1). The co-treatment of slices with 0.5 µM of DPDS significantly attenuated the MeHg-induced reduction in oxygen consumption reaching levels indistinguishable from those in mitochondria isolated from control liver slices ($p<0.005$, Table 1). At 1 and 5 µM of DPDS the MeHg-induced reduction in oxygen consumption was restored to normal levels; however, the protection was partial (oxygen consumption was similar to control values, but was not different from that of MeHg group; Table 1).

**Discussion**

This study investigated the efficacy of DPDS in attenuating MeHg-induced hepatic mitochondrial dysfunction. Our findings demonstrated that MeHg caused mitochondrial dysfunction as observed by a decrease in mitochondrial metabolic function, an increase in

ROS production, a decrease in the oxygen consumption and the collapse of the mitochondrial ΔΨm (Scheme 2). These findings are in agreement with other studies corroborating mitochondrial dysfunction after exposure to MeHg both *in vivo* and *in vitro* (Cambier et al., 2009; Glaser et al., 2010; Mori et al., 2007; Roos et al., 2011; Wagner et al., 2010; Yin et al., 2011).

MeHg is known to alter mitochondrial $Ca^{2+}$ regulation, causing increased mitochondrial $Ca^{2+}$ transient and the MeHg *per se* inducing MTP opening (Atchison and Hare, 1994). Opening of the MTP allows the passive diffusion of low molecular weight molecules, including $Ca^{2+}$, through the internal mitochondrial membrane, thus dissipating the proton gradient across the mitochondrial membrane. In this way, MeHg can cause mitochondrial dysfunction and lead to increased ROS production (Aschner et al., 2007). Given these observations, results presented herein are consistent with MeHg-induced decreased the mitochondrial ΔΨm that is secondary to $Ca^{2+}$ entry during slice pre-incubation and the ensuing overproduction of ROS and MTP opening.

Overall, DPDS prevented the MeHg-induced mitochondrial toxicity in liver slices. The reduction of MTT to formazan, catalyzed by mitochondrial dehydrogenase enzymes, is indicative of the activity of these enzymes. Accordingly, one potential explanation for the efficacy of DPDS in protecting against MeHg-induced mitochondrial toxicity may reside in its ability to restore the activity of mitochondrial dehydrogenase enzymes possibly by forming a complex with MeHg thus effectively reducing total cellular MeHg burden (Figure 1). Our results are consistent with previous *in vivo* works in which MeHg exposure decreased the activity of dehydrogenases enzymes (Glaser et al., 2010; Mori et al., 2011; Yoshino et al., 1966). Dehydrogenase enzymes inhibition by MeHg could be directly related to covalent modification of the enzyme by the formation of MeHg-protein adducts (Glaser et al., 2010).

Corroborating the inhibition of dehydrogenases by MeHg, mitochondrial oxygen consumption was decreased after exposure of liver slices to MeHg (Table 1). As observed in the MTT assay, DPDS partially (at 1 and 5 µM) or completely (at 0.5 µM) prevented the MeHg-induced inhibition of mitochondrial oxygen consumption. Accordingly, we posit that the MeHg-induced inhibition of oxygen consumption and its partial or complete normalization by DPDS is associated with modulation of respiratory chain mitochondrial dehydrogenases.

DPDS also prevented the MeHg-induced decrease in mitochondrial ΔΨm, although only at low concentrations (Figure 4A,B). The highest concentration of DPDS only partially protected from the MeHg-induced mitochondrial depolarization (Figure 4C). In fact at 5 µM

DPDS caused mitochondrial depolarization, likely related to excessive oxidation of critical thiol groups on mitochondrial proteins (Puntel et al., 2010).

In the case of MeHg-induced ROS formation, DPDS partially or completely prevented the DCFH oxidation (Figure 3). Furthermore, as observed for mitochondrial polarization, DPDS caused a concentration-dependent increase in DCFH oxidation, indicating that concentrations higher than 1 μM can be oxidizing important thiol groups from mitochondrial proteins and disrupting mitochondrial integrity.

In summary, MeHg can either deplete glutathione (GSH) levels which induce oxidative stress and lipid peroxidation, or directly interact with sulfhydryl proteins, leading to protein oxidation and loss of function, both the events can lead to mitochondrial dysfunction (Scheme 2). DPDS can decrease MeHg toxicity to mitochondria via its reduction to selenol/selenolate (PhSeH/PhSe−), which can directly block the pro-oxidative effects of MeHg due to its thiol-peroxidase activity or can form a stable complex with MeHg (see Scheme 1 and 2). However, at high concentrations the toxic effects of DPDS should be considered, since it can cause the oxidation of -SH groups leading to the inactivation of important thiol-containing proteins (see Scheme 1).

In conclusion, results presented here demonstrate that DPDS at low concentrations can prevent the mitochondrial dysfunction induced by MeHg. The efficacy of DPDS in protecting against MeHg-induced mitochondrial toxicity could be, at least in part, attributed to the interaction of its PhSeH/PhSe− with MeHg to form a stable complex. MeHg toxicity is also associated with overproduction of mitochondrial ROS. Accordingly, an alternative and/or supplementary mode of action invokes the antioxidant properties of DPDS and modulation of oxidative stress in ameliorating the mitochondrial effects of MeHg. Future studies should provide additional pharmacological characterization of DPDS and determine the mechanism(s) responsible for its protection and its efficacy in treatment broad spectrum disorders with inherent mitochondrial dysfunction.

**Acknowledgments**

Supported by FAPERGS (Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), FINEP (Rede Instituto Brasileiro de Neurociência (IBN-Net) # 01.06.0842-00), FAPERGS-PRONEX-CNPQ and INCT-EN (Instituto Nacional de Ciência e Tecnologia em Excitotoxicidade e Neuroproteção). Aschner was supported by a grant from the US National Institutes of Health, ES R01 07331.

**References**

Allan, C.B., Lacourciere, G.M., Stadtman, T.C., 1999. Responsiveness of selenoproteins to dietary selenium. Annu. Rev. Nutr. 19, 1–16.

Aschner, M., Syversen, T., Souza, D.O., Rocha, J.B.T., Farina, M., 2007. Involvement of glutamate and reactive oxygen species in methylmercury neurotoxicity. Braz. J. Med. Biol. Res. 40, 285–291.

Atchison, W.D., Hare, M.F., 1994. Mechanisms of methylmercury-induced neurotoxicity. FASEB J. 8, 622–629.

Brustovetsky, N., Dubinsky, J.M., 2000. Dual responses of CNS mitochondria to elevated calcium. J. Neurosci. 20, 103–113.

Burger, M., Fachinetto, R., Calegari, L., Paixao, M.W., Braga, A.L., Rocha, J.B.T., 2004. Effects of age on reserpine-induced orofacial dyskinesia and possible protection of diphenyl diselenide. Brain Res. Bull. 64, 339–345.

Burger, M.E., Fachinetto, R., Wagner, C., Perottoni, J., Pereira, R.P., Zeni, G., Rocha, J.B.T., 2006. Effects of diphenyl-diselenide on orofacial dyskinesia model in rats. Brain Res. Bull. 70, 165–170.

Cambier, S., Benard, G., Mesmer-Dudons, N., Gonzalez, P., Rossignol, R., Brethes, D., Bourdineaud, J.P., 2009. At environmental doses, dietary methylmercury inhibits mitochondrial energy metabolism in skeletal muscles of the zebra fish (Danio rerio). Int. J. Biochem. Cell Biol. 41, 791–799.

Ceccatelli, S., Dare, E., Moors, M., 2010. Methylmercury-induced neurotoxicity and apoptosis. Chem. Biol. Interact. 188, 301–308.

Choi, A.L., Budtz-Jorgensen, E., Jorgensen, P.J., Steuerwald, U., Debes, F., Weihe, P., Grandjean, P. 2008. Selenium as a potential protective factor against mercury developmental neurotoxicity. Environ. Res. 107, 45–52.

Clarkson, T.W., Magos, L., Myers, G.J., 2003. The toxicology of mercury - Current exposures and clinical manifestations. N. Engl. J. Med. 349, 1731–1737.

de Freitas, A.S., Funck, V.R., Rotta, M.D., Bohrer, D., Morschbacher, V., Puntel, R.L., Nogueira, C.W., Farina, M., Aschner, M., Rocha, J.B.T., 2009. Diphenyl diselenide, a simple organoselenium compound, decreases methylmercury-induced cerebral, hepatic and renal oxidative stress and mercury deposition in adult mice. Brain Res. Bull. 79, 77–84.

de Freitas, A.S., Prestes, A.S., Wagner, C., Sudati, J.H., Alves, D., Porciuncula, L.O., Kade, I.J., Rocha, J.B.T., 2010. Reduction of diphenyl diselenide and analogs by mammalian

thioredoxin reductase is independent of their gluthathione peroxidase-like activity: a possible novel pathway for their antioxidant activity. Molecules 15, 7699–7714.

de Freitas, A.S., Rocha, J.B.T., 2011. Diphenyl diselenide and analogs are substrates of cerebral rat thioredoxin reductase: A pathway for their neuroprotective effects. Neurosci Lett. In press.

Dorea, J.G., Barbosa, A.C., Silva, G.S., 2006. Fish mercury bioaccumulation as a function of feeding behavior and hydrological cycles of the Rio Negro, Amazon. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 142, 275–283.

Farina, M., Campos, F., Vendrell, I., Berenguer, J., Barzi, M., Pons, S., Sunol, C., 2009. Probucol increases glutathione peroxidase-1 activity and displays long-lasting protection against methylmercury toxicity in cerebellar granule cells. Toxicol. Sci. 112, 416-426.

Farina, M., Aschner, M., Rocha, J.B.T., 2011a. Oxidative stress in MeHg-induced neurotoxicity. Toxicol. Appl. Pharmacol. In press.

Farina, M., Rocha, J.B.T., Aschner, M., 2011b. Mechanisms of methylmercury-induced neurotoxicity: Evidence from experimental studies. Life Sci. In press.

Flohe, L., 1988. Glutathione peroxidase. Basic Life Sci. 49, 663–668.

Fredriksson, A., Gardlund, A.T., Bergman, K., Oskarsson, A., Ohlin, B., Danielsson, B., Archer, T., 1993. Effects of maternal dietary supplementation with selenite on the postnatal-development of rat offspring exposed to methyl mercury in-utero. Pharmacol. Toxicol. 72, 377–382.

Funchal, C., Moretto, M.B., Vivian, L., Zeni, G., Rocha, J.B.T., Pessoa-Pureur, R., 2006. Diphenyl ditelluride- and methylmercury-induced hyperphosphorilation of the high molecular weight neurofilament subunit is prevented by organoselenium compounds in cerebral cortex of young rats. Toxicology 222, 143–153.

Ganther, H.E., 1971. Reduction of selenotrisulfide derivative of glutathione to a persulfide analog by glutathione reductase. Biochemistry 10, 4089–4098.

Garcia-Ruiz, C., Colell, A., Mari, M., Morales, A., Fernández-Checa, J.C., 1997. Direct effect of ceramide on the mitochondrial electron transport chain leads to generation of reactive oxygen species - Role of mitochondrial glutathione. J. Biol. Chem. 272, 11369–11377.

Ghisleni, G., Porciuncula, L.O., Cimarosti, H., Batista, T.R.J., Salbego, C.G., Souza, D.O., 2003. Diphenyl diselenide protects rat hippocampal slices submitted to oxygen-

glucose deprivation and diminishes inducible nitric oxide synthase immunocontent. Brain Res. 986, 196–199.

Glaser, V., Nazari, E.M., Muller, Y.M., Feksa, L., Wannmacher, C.M., Rocha, J.B.T., de Bem, A.F., Farina, M., Latini, A., 2010. Effects of inorganic selenium administration in methylmercury-induced neurotoxicity in mouse cerebral cortex. Int. J. Dev. Neurosci. 28, 631–637.

Guo, Q., Christakos, S., Robinson, N., Mattson, M.P., 1998. Calbindin D28k blocks the proapoptotic actions of mutant presenilin 1: reduced oxidative stress and preserved mitochondrial function. Proc. Natl. Acad. Sci. U S A 95, 3227–3232.

Halliwell, B., Gutteridge, J.M.C., 1999. Free radicals in biology and medicine, fourth ed. Oxford University Press, New York.

Holmgren, A., 1989. Thioredoxin and glutaredoxin systems. J. Biol. Chem. 264, 13963–13966.

Lando, A.M., Zhang, Y., 2011. Awareness and knowledge of methylmercury in fish in the United States. Environ. Res. 111, 442–450.

Limke, T. L., Atchison, W.D., 2002. Acute exposure to methylmercury opens the mitochondrial permeability transition pore in rat cerebellar granule cells. Toxicol. Appl. Pharmacol. 178, 52–61.

Limke, T.L., Otero-Montanez, J.K.L., Atchison, W.D., 2003. Evidence for interactions between intracellular calcium stores during methylmercury-induced intracellular calcium dysregulation in rat cerebellar granule neurons. J. Pharmacol. Exp. Ther. 304, 949–958.

Limke, T.L., Heidemann, S.R., Atchison, W.D., 2004. Disruption of intraneuronal divalent cation regulation by methylmercury: are specific targets involved in altered neuronal development and cytotoxicity in methylmercury poisoning? Neurotoxicology 25, 741–760.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Moretto, M.B., Funchal, C., Zeni, G., Pessoa-Pureur, R., Rocha, J.B.T., 2005. Selenium compounds prevent the effects of methylmercury on the in vitro phosphorylation of cytoskeletal proteins in cerebral cortex of young rats. Toxicol. Sci. 85, 639–646.

Mori, N., Yasutake, A., Hirayama, K., 2007. Comparative study of activities in reactive oxygen species production/defense system in mitochondria of rat brain and liver, and their susceptibility to methylmercury toxicity. Arch. Toxicol. 81, 769–776.

Mori, N., Yasutake, A., Marumoto, M., Hirayama, K., 2011. Methylmercury inhibits electron transport chain activity and induces cytochrome c release in cerebellum mitochondria. J. Toxicol. Sci. 36, 253–259.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Mugesh, G., Panda, A., Singh, H.B., Punekar, N.S., Butcher, R.J., 2001. Glutathione peroxidase-like antioxidant activity of diaryl diselenides: a mechanistic study. J. Am. Chem. Soc. 123, 839–850.

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

Nogueira, C.W., Rocha, J.B.T., 2010. Diphenyl diselenide a janus-faced molecule. J. Braz. Chem. Soc. 21, 2055–2071.

Nogueira, C.W., Rocha, J.B.T., 2011. Toxicology and pharmacology of selenium: emphasis on synthetic organoselenium compounds. Arch. Toxicol. In press.

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

Paulmier, C., 1986. Selenoorganic functional groups, in: Selenium reagents and intermediates in organic synthesis. Pergamon Press, Oxford, pp. 25-51.

Pinheiro, M.C.N., do Nascimento, J.L.M., Silveira, L.C.L., da Rocha, J.B.T., Aschner, M., 2009. Mercury and selenium - A review on aspects related to the health of human populations in the Amazon. Environ. Bioind. 4, 222–245.

Puntel, R.L., Roos, D.H., Folmer, V., Nogueira, C.W., Galina, A., Aschner, M., Rocha, J.B.T., 2010. Mitochondrial dysfunction induced by different organochalchogens is mediated by thiol oxidation and is not dependent of the classical mitochondrial permeability transition pore opening. Toxicol. Sci. 117, 133–143.

Roos, D.H., Puntel, R.L., Farina, M., Aschner, M., Bohrer, D., Rocha, J.B.T., Barbosa, N.B.V., 2011. Modulation of methylmercury uptake by methionine: prevention of mitochondrial dysfunction in rat liver slices by a mimicry mechanism. Toxicol. Appl. Pharmacol. 252, 28–35.

Valko, M., Morris, H., Cronin, M.T., 2005. Metals, toxicity and oxidative stress. Curr. Med. Chem. 12, 1161–1208.

Wagner, C., Vargas, A.P., Roos, D.H., Morel, A.F., Farina, M., Nogueira, C.W., Aschner, M., Rocha, J.B.T., 2010. Comparative study of quercetin and its two glycoside derivatives

quercitrin and rutin against methylmercury (MeHg)-induced ROS production in rat brain slices. Arch. Toxicol. 84, 89–97.

Yin, Z., Lee, E., Ni, M., Jiang, H., Milatovic, D., Rongzhu, L., Farina, M., Rocha, J.B.T., Aschner, M., 2011. Methylmercury-induced alterations in astrocyte functions are attenuated by ebselen. Neurotoxicology 32, 291–299.

Yoshino, Y., Mozai, T., Nakao, K., 1966. Biochemical changes in the brain in rats poisoned with an alkymercury compound, with special reference to the inhibition of protein synthesis in brain cortex slices. J. Neurochem. 13, 1223–1230.

**Legends of Schemes**

**Scheme 1. Possible pathways for the toxic and therapeutic effects of DPDS.** In the toxic pathway, PhSeH formation leads to the oxidation of free- or protein-thiols, causing GSH depletion and protein loss-of-function. In the therapeutic pathway, DPDS decomposes peroxides either as a substrate for TrxR or as a mimic of GPx via the formation of the PhSeH/PhSe− intermediate. DPDS can decrease MeHg toxicity via its reduction to PhSeH/PhSe−, forming a stable complex with MeHg (PhSeHgMe).

**Scheme 2. Protective effect of DPDS against MeHg-induced mitochondrial dysfunction.** MeHg can either deplete GSH levels which exacerbate oxidative stress and lipid peroxidation, or directly interact with sulfhydryl proteins, leading to protein oxidation and loss-of-function. These events can reduce oxygen consumption and collapse the mitochondrial ΔΨm. DPDS can decrease mitochondrial MeHg toxicity via its transformation to PhSeH/PhSe−, which can directly block the pro-oxidative effects of MeHg due to its thiol-peroxidase activity or secondary to the formation of a stable complex with MeHg.

**Legends of Figures**

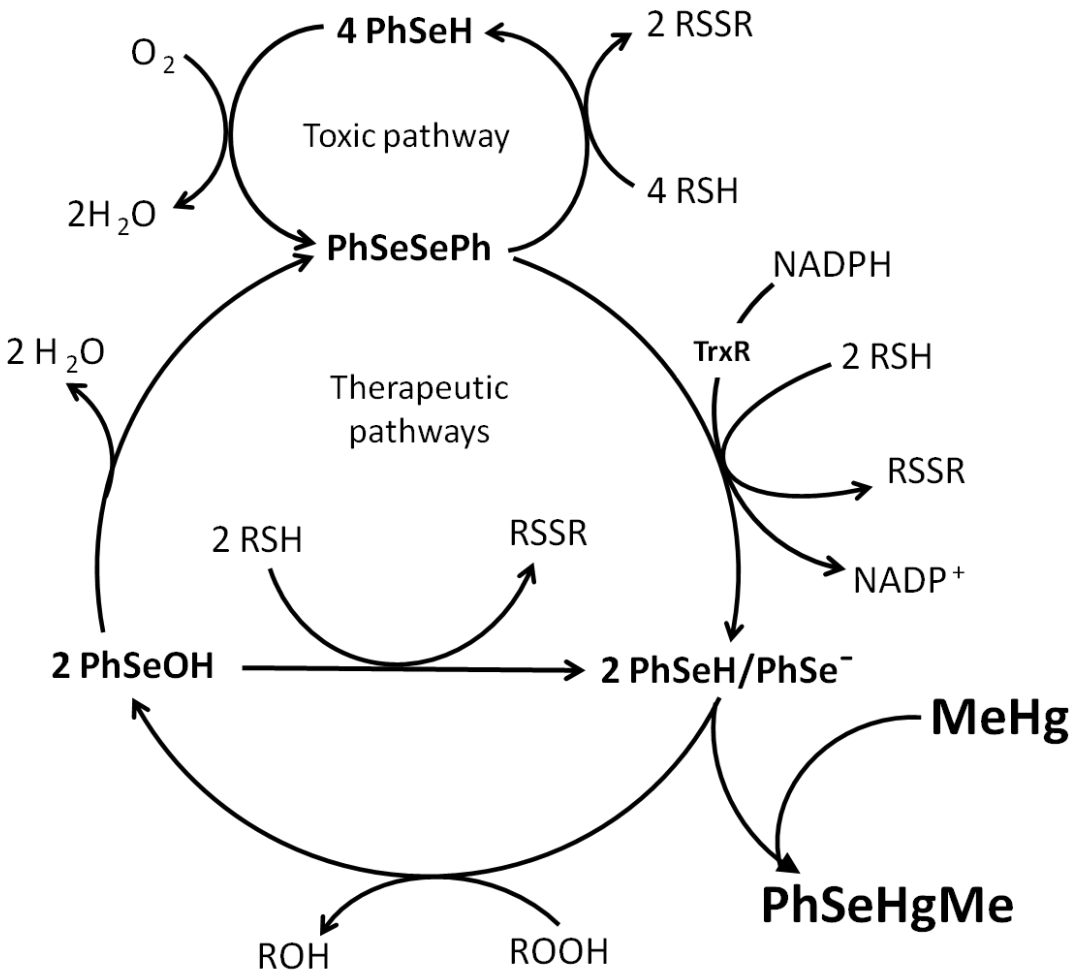
**Figure 1. Effect of pre-treatment of liver slices with MeHg (25 µM) and/or DPDS (0.5, 1, 5 µM) for 30 min on isolated mitochondrial metabolic function (MTT reduction).** Data are expressed as the mean ± S.E.M., n=3–4. Experiments were performed in duplicates. (*) represents $p<0.05$ as compared to control group by Duncan's multiple range test. (#) represents $p<0.05$ as compared to MeHg group by Duncan's multiple range test

**Figure 2. Effect of pre-treatment of liver slices with MeHg (25 µM) on isolated mitochondrial ROS generation.** (A) Oxidation of $H_2$-DCFH by mitochondria isolated from slices previously incubated with MeHg and/or DPDS. (B) Statistical analysis of the 300 s timepoint. $H_2$-DCFDA (1 µM) was added at 15 s of recording. $CaCl_2$ (60 µM) was added at 30 s of recording. Data are expressed as the mean ± S.E.M., n=3–4. Experiments were performed in duplicates. Bars with different letters are significantly different ($P<0.05$) from each other.

**Figure 3. Effect of pre-treatment of liver slices with MeHg (25 µM) and/or DPDS (0.5, 1, 5 µM) on mitochondrial ROS generation.** (A) Oxidation of $H_2$-DCFH by mitochondria isolated from slices previously incubated with MeHg and/or DPDS. (B) Statistical analysis of the 300 s timepoint. $H_2$-DCFDA (1 µM) was added at 15 s of recording. Data are expressed as the mean ± S.E.M., n=3–4. Experiments were performed in duplicates. Bars with different letters are significantly different ($P<0.05$) from each other.

**Figure 4. Effects of pre-treatment of liver slices with MeHg (25 µM) and/or DPDS 0.5 (A), 1 (B), 5 µM (C) on $\Delta\Psi m$.** The mitochondria and FCCP were added where indicated by arrows. Mitochondria (1 mg/ ml) were added at 15 s of recording. FCCP (1 µM) was added at 200 s of recording. The traces are representative of 3 independent experiments.

**Scheme 1.**


4 PhSeH
2 RSSR
O 2
Toxic pathway
4 RSH
2H 2O
PhSeSePh
NADPH
TrxR
2 RSH
2 H 2O
Therapeutic
pathways
RSSR
2 RSH
RSSR
NADP+
2 PhSeOH
2 PhSeH/PhSe−
MeHg
PhSeHgMe
ROH
ROOH

**Scheme 2.**

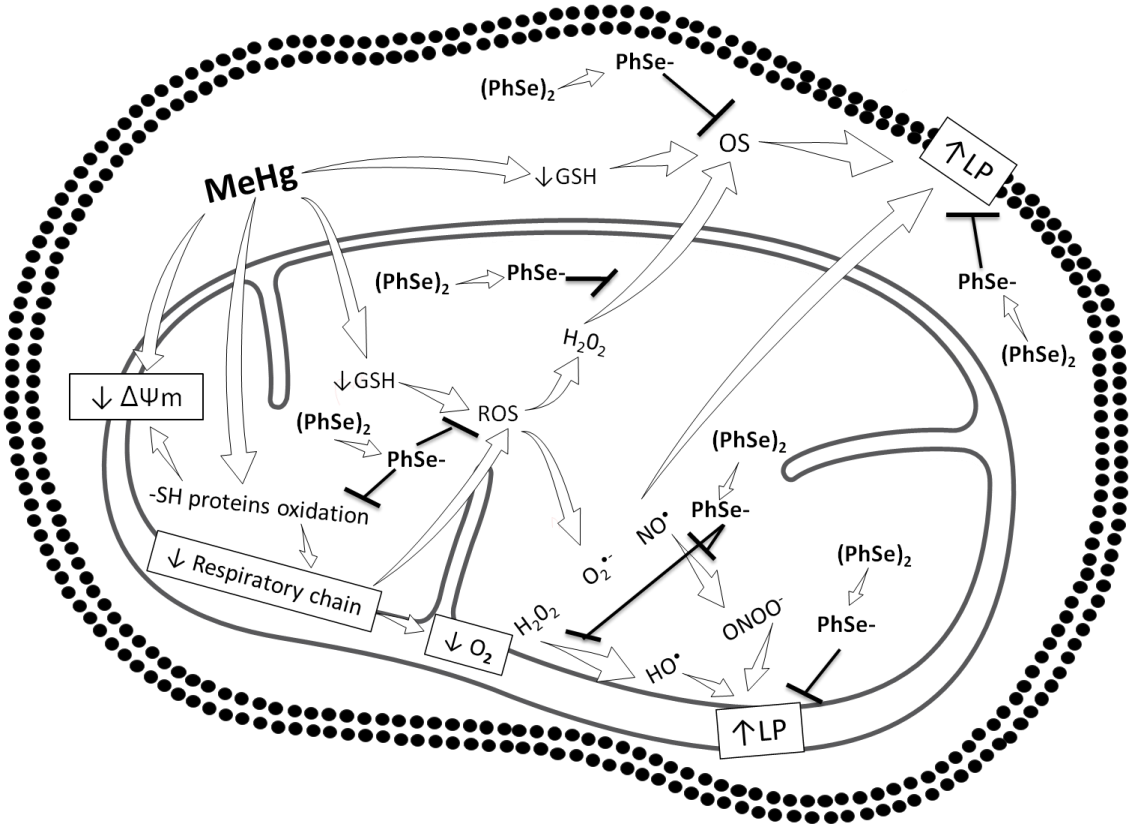

(PhSe)2
PhSe-
OS
↑LP
MeHg
↓GSH
(PhSe)2
PhSe-
PhSe-
(PhSe)2
H2O2
↓ ΔΨm
↓GSH
ROS
(PhSe)2
PhSe-
(PhSe)2
-SH proteins oxidation
NO•
PhSe-
O2•-
(PhSe)2
↓ Respiratory chain
H2O2
ONOO-
PhSe-
↓ O2
HO•
↑LP

**Table 1**

Effects of MeHg (25 µM) and DPDS on mitochondrial respiratory rates.

| | Respiration rate (nmol $O_2$/ml/min) | Respiration rate with succinate (nmol $O_2$/ml/min) |
|---|---|---|
| Control | 1,11 ± 0,12 | 3,7 ± 0,84 |
| DPDS 0.5 µM | 1,36 ± 0,18 | 3,42 ± 0,77 |
| DPDS 1 µM | 1,17 ± 0,12 | 3,64 ± 0,39 |
| DPDS 5 µM | 1,23 ± 0,14 | 3,22 ± 0,56 |
| MeHg | 1,05 ± 0,12 | 2,45 ± 0,63[ab] |
| DPDS 0.5 µM + MeHg | 1,08 ± 0,08 | 4,55 ± 0,48 |
| DPDS 1 µM + MeHg | 1,25 ± 0,18 | 3,39 ± 0,37 |
| DPDS 5 µM + MeHg | 1,08 ± 0,13 | 3,44 ± 0,87 |

Oxygen consumption was monitored in the presence of 5 mM succinate as a respiratory substrate. Values are the mean ± S.D. of 3 different experiments.

[a] Indicates $p<0.05$ as compared to control.

[b] Indicates $p<0.05$ as compared to DPDS 0.5 µM + MeHg.

**Figure 1.**

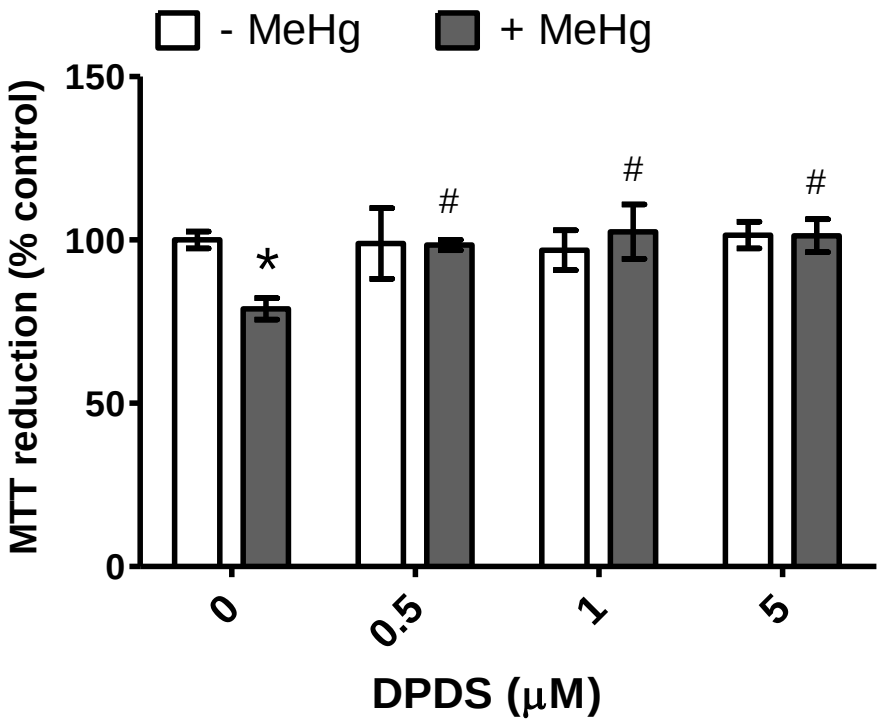


**Figure 2.**

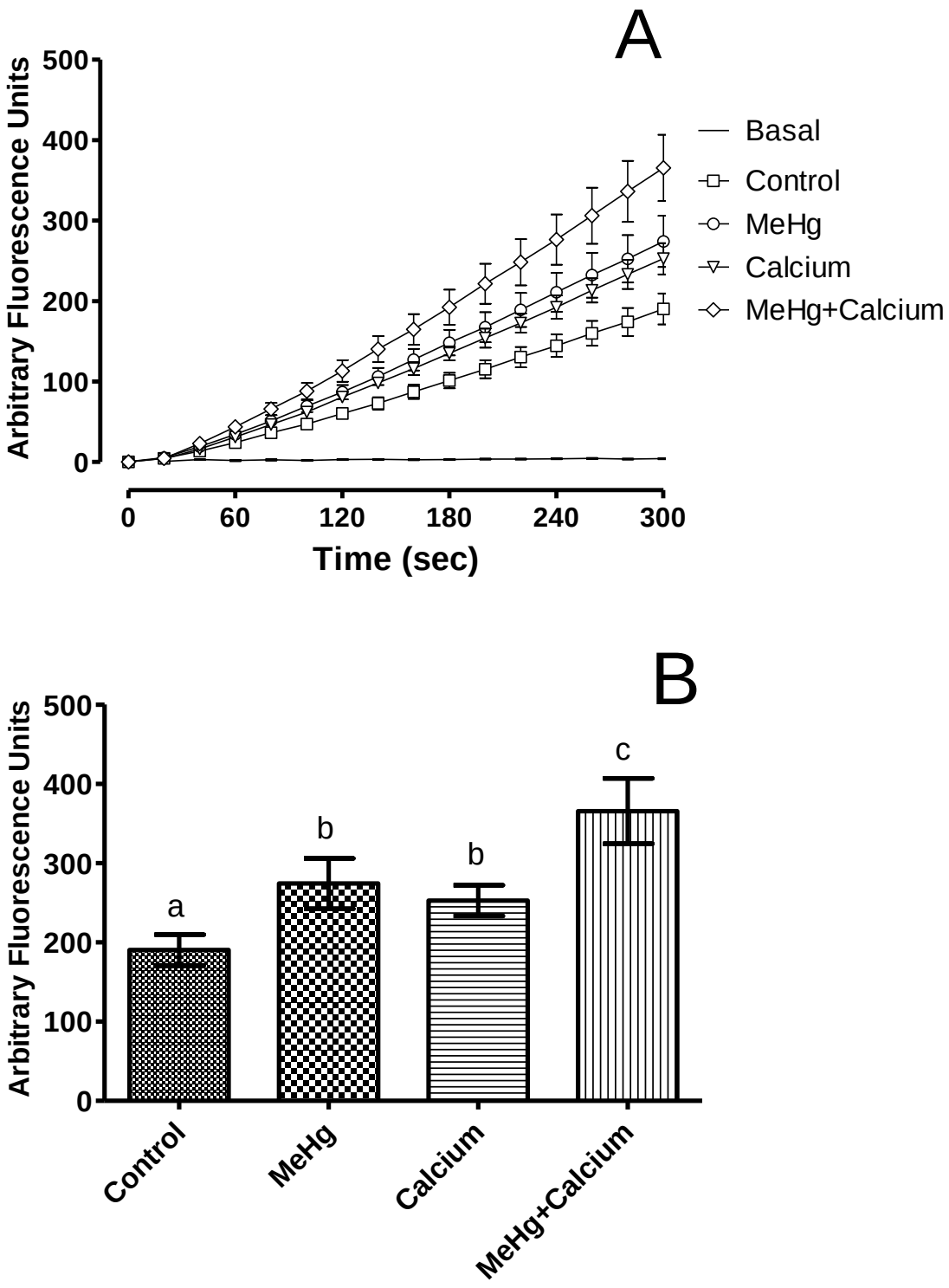

A
Arbitrary Fluorescence Units
500
400
300
200
100
0
0
60
120
180
240
300
Time (sec)
Basal
Control
MeHg
Calcium
MeHg+Calcium
B
Arbitrary Fluorescence Units
500
400
300
200
100
0
a
b
b
c
Control
MeHg
Calcium
MeHg+Calcium

**Figure 3.**

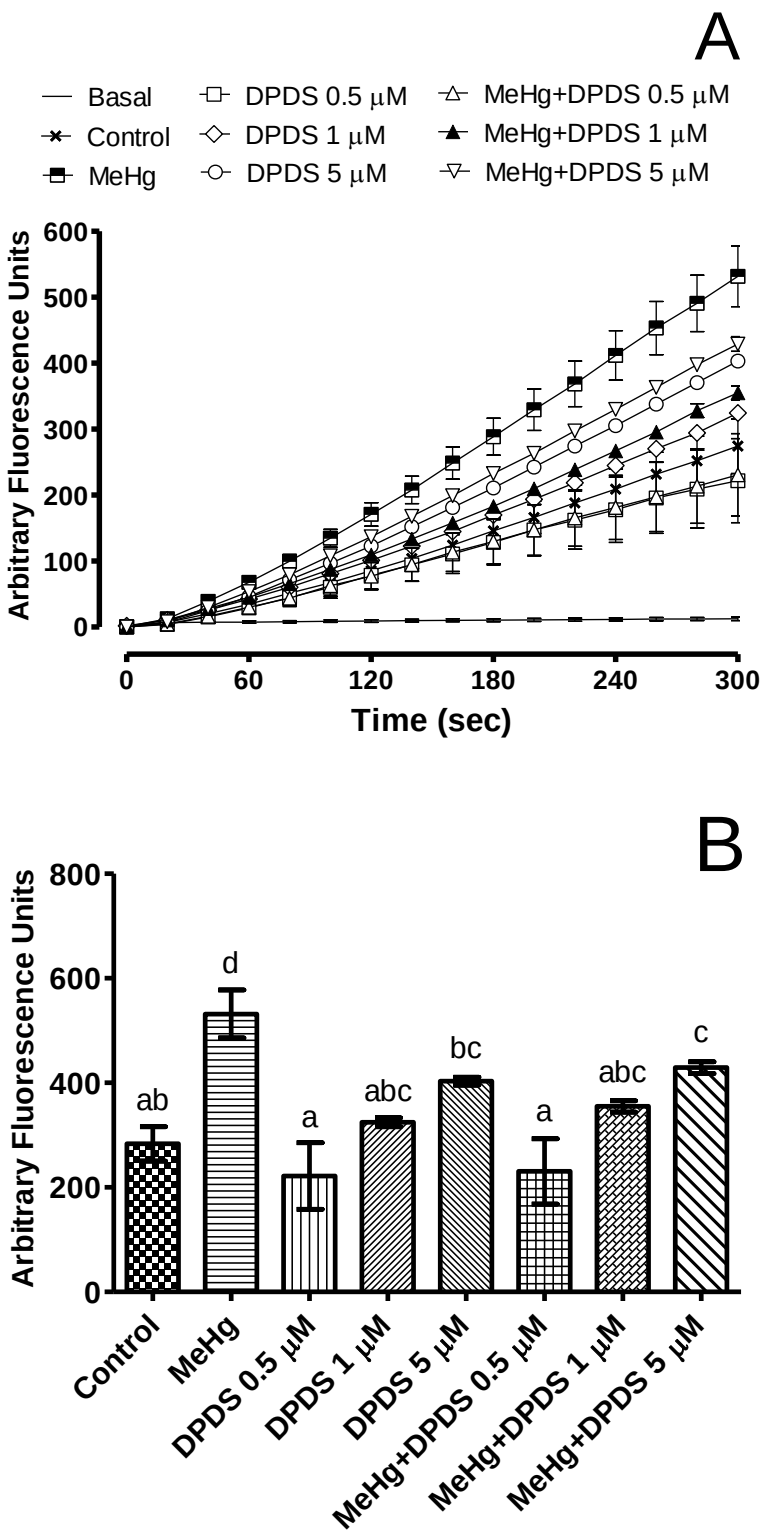
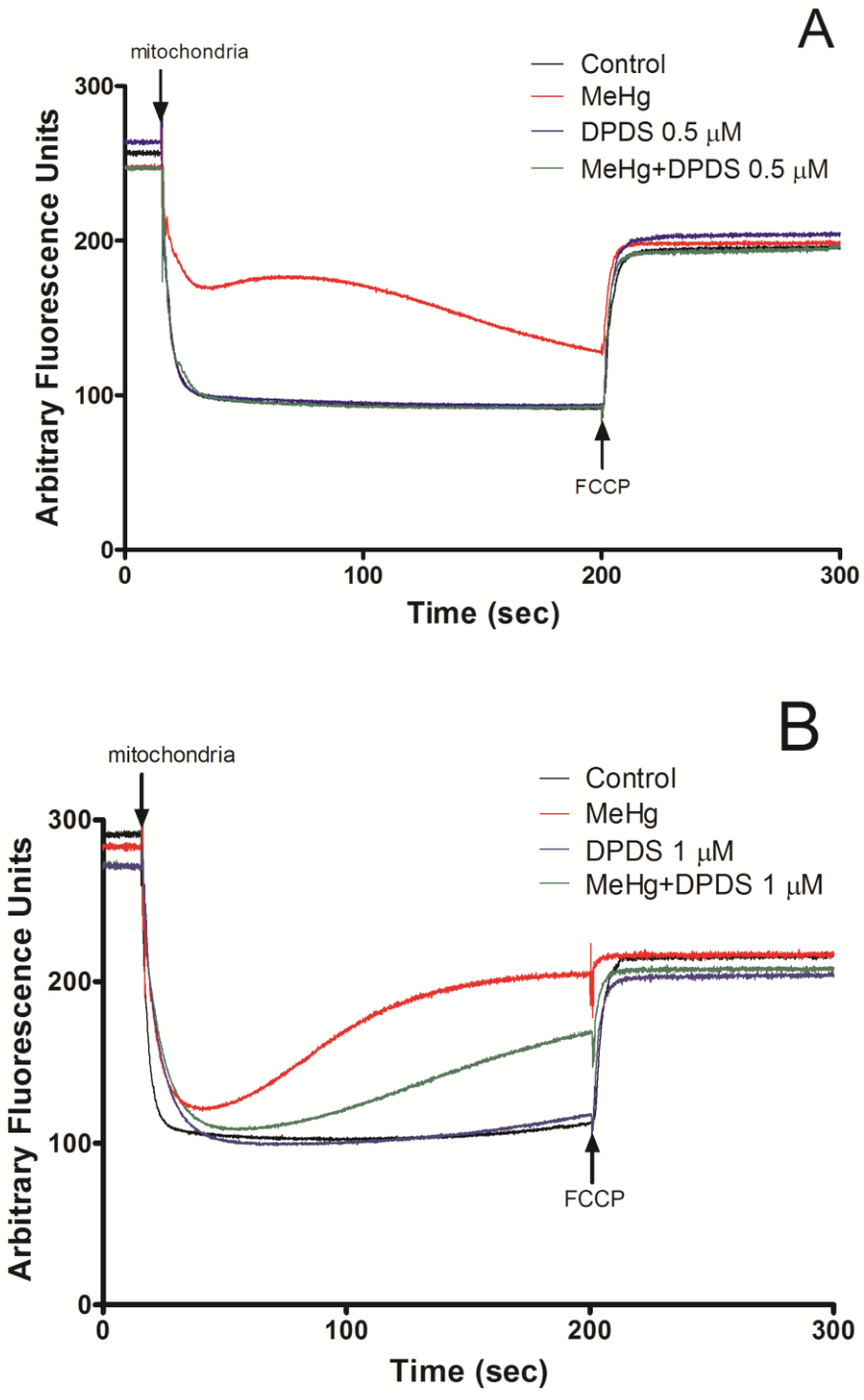
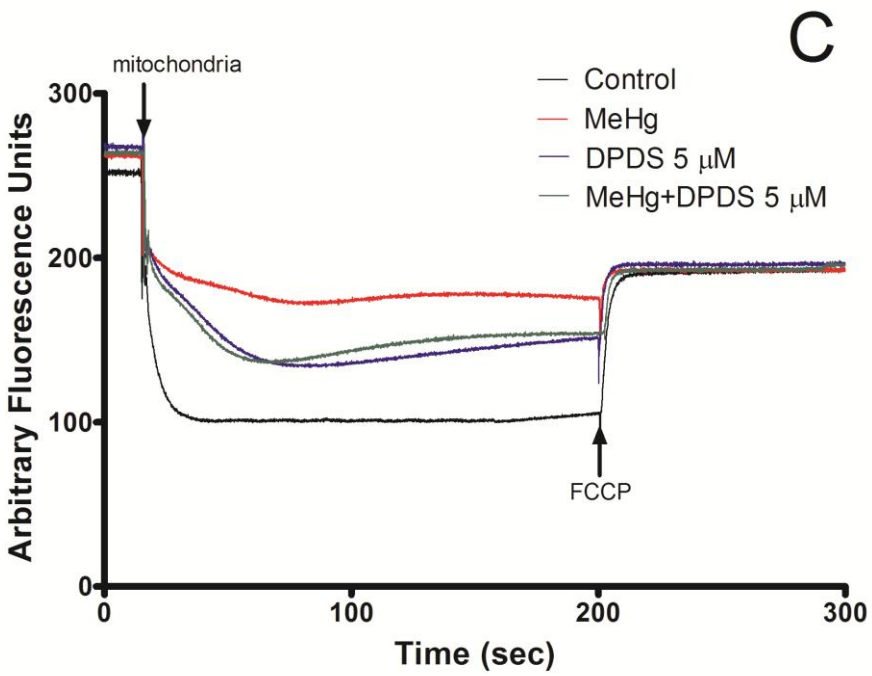

A
Basal
Control
MeHg
DPDS 0.5 μM
DPDS 1 μM
DPDS 5 μM
MeHg+DPDS 0.5 μM
MeHg+DPDS 1 μM
MeHg+DPDS 5 μM
Arbitrary Fluorescence Units
600
500
400
300
200
100
0
0
60
120
180
240
300
Time (sec)
B
Arbitrary Fluorescence Units
800
600
400
200
0
ab
d
a
abc
bc
a
abc
c
Control
MeHg
DPDS 0.5 μM
DPDS 1 μM
DPDS 5 μM
MeHg+DPDS 0.5 μM
MeHg+DPDS 1 μM
MeHg+DPDS 5 μM

**Figure 4.**


A
mitochondria
Control
MeHg
DPDS 0.5 μM
MeHg+DPDS 0.5 μM
Arbitrary Fluorescence Units
300
200
100
0
FCCP
0
100
200
300
Time (sec)
B
mitochondria
Control
MeHg
DPDS 1 μM
MeHg+DPDS 1 μM
Arbitrary Fluorescence Units
300
200
100
0
FCCP
0
100
200
300
Time (sec)


C
mitochondria
Control
MeHg
DPDS 5 μM
MeHg+DPDS 5 μM
Arbitrary Fluorescence Units
300
200
100
0
FCCP
0
100
200
300
Time (sec)

## 2.3. O DISSELENETO DE DIFENILA AUMENTA A NEUROTOXICIDADE INDUZIDA POR METILMERCÚRIO EM RATOS

**Manuscrito 2**

### DIPHENYL DISELENIDE INCREASES METHYLMERCURY-INDUCED NEUROTOXICITY IN RATS

CRISTIANE L. DALLA CORTE, CAROLINE WAGNER, JÉSSIE H. SUDATI, BRUNA COMPARSI, GERLÂNIA O. LEITE, ALCINDO BUSANELLO, JOÃO B. T. ROCHA

**Diphenyl diselenide increases methylmercury-induced neurotoxicity in rats**

Cristiane L. Dalla Corte[a], Caroline Wagner[b], Jéssie H. Sudati[a], Bruna Comparsi[a], Gerlânia O. Leite[c], Alcindo Busanello[a], João B. T. Rocha[a]*

[a] *Departamento de Química, Programa de Pós-graduação em Ciências Biológicas: Bioquímica Toxicológica, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Cep 97105-900, Santa Maria, RS, Brazil.*

[b] *Universidade Federal do Pampa – Campus Caçapava do Sul, Av. Pedro Anunciação, Vila Batista, Cep 96570-000, Caçapava do Sul, RS, Brazil.*

[c] *Universidade Regional do Cariri, Laboratório de Farmacologia e Química Molecular, Rua Cel. Antônio Luís 1161, Cep 63100-000, Crato, CE, Brazil.*

[*] *Corresponding author:*
*João Batista Teixeira da Rocha*
*UFSM – CCNE – Dep. de Química*
*Cep 97105-900, Santa Maria, RS, Brasil.*
*Tel: #55-55-3220-8140*
*Fax: #55-55-3220-8978*
E-mail: jbtrocha@yahoo.com.br

**Abstract**

Methylmercury (MeHg) is an important environmental contaminant with toxic effects to humans and experimental animals. Diphenyl diselenide (DPDS) is an organoselenium compound that has been extensively studied for its potential antioxidant, pharmacological and neuroprotective activities. In this study we attempted to investigate the efficacy of DPDS in attenuating MeHg toxicity in rats. Rats were treated with MeHg (5 mg/ kg/ day, i.g.) and/ or DPDS (1 mg/ kg /day, i.p.) during 21 days. Body weight gain and motor deficits (rotarod and open-field tests) were evaluated during the treatment. In addition, hepatic and cerebral mitochondrial dysfunction (reactive oxygen species (ROS) formation, total and non-protein thiol levels, mitochondrial membrane potential ($\Delta\Psi m$), mitochondrial metabolic function and mitochondrial swelling), hepatic, cerebral and muscular mercury (Hg) levels, and hepatic, cerebral and renal TrxR activity were evaluated in rats treated with MeHg and/ or DPDS. MeHg caused a decrease in body weight and induced motor deficits in rats. MeHg also induced hepatic and cerebral mitochondrial dysfunction, and inhibited TrxR activity in liver, brain and kidney. The co-treatment with DPDS protected hepatic and cerebral mitochondrial thiols from depletion by MeHg but, did not prevent hepatic and cerebral mitochondrial dysfunction, nor recovered hepatic, cerebral and renal TrxR activity inhibited by MeHg. Additionally, the co-treatment with DPDS increased Hg accumulation in liver, brain and skeletal muscle, and increased the motor deficits and body weight lost. In conclusion, the results of the current study indicate that DPDS, at a low dose, can increase Hg deposition and the neurotoxic effects induced by MeHg exposure in rats.

**Keywords:** Methylmercury, organoselenium compounds, oxidative stress, thioredoxin reductase, mitochondrial dysfunction.

**Introduction**

Methylmercury (MeHg) is one of the most dangerous environmental contaminant with toxic effects to humans and experimental animals (Carvalho et al., 2011; Clarkson et al., 2003). Environmental MeHg is derived from inorganic mercury biomethylation carried out primarily by aquatic microorganisms (Compeau and Bartha, 1985) with subsequent accumulation in the marine food chain (Dorea, 2008; Kuntz et al., 2009; Shanker et al., 2004). Fish and seafood are the most prevalent sources of human exposure to MeHg, and many populations are exposed to potentially dangerous levels of MeHg in their diet (WHO). MeHg causes acute and chronic damage to multiple organs, most profoundly to the central nervous system (CNS) in particular when exposures occur during the initial stages of brain development (Clarkson et al., 2003; Costa et al., 2004; Grandjean and Herz, 2011; Hassan et al., 2011; Johansson et al., 2007; Marsh et al., 1995).

The events mediating MeHg toxicity are largely dependent upon its electrophilic properties, which allows for its interaction with soft nucleophilic groups (mainly thiols and selenols) from either low or high-molecular-weight biomolecules (Farina et al., 2011a). The interaction of MeHg with soft nucleophilic groups from biomolecules is responsible, at least in part, for decreased antioxidant capacity and increased reactive oxygen species (ROS) generation (Aschner et al., 2010; Farina et al., 2009; Farina et al., 2011a; Franco et al., 2007; Kaur et al., 2006). Most important MeHg can disrupt the activity of thiol- and selenol-containing proteins, such as glutathione peroxidase (GPx), thioredoxin (Trx) and thioredoxin reductase (TrxR) (Branco et al., 2011; Carvalho et al., 2008, 2011; Farina et al., 2009; Franco et al., 2009; Glaser et al., 2010a; Wagner et al., 2010a,b). These proteins are important components of the cellular antioxidant system, and their inhibition contributes to the disruption of the normal redox balance of cells (Farina et al., 2011a). Mammalian TrxR is a selenoenzyme containing a unique, catalytically-active selenolthiol/selenenylsulfide in the conserved C-terminal sequence (-Gly-Cys- Sec-Gly; (Sandalova et al., 2001; Zhong et al., 2000). The thioredoxin (Trx) system, composed of thioredoxin reductase (TrxR), Trx, and NADPH, is critical for the cellular stress response, protein repair, and protection against oxidative damage (Arner and Holmgren, 2000; Lillig and Holmgren, 2007).

In addition, MeHg can disrupt mitochondrial functioning by targeting specific thiol-containing proteins, including respiratory chain complexes (Atchison and Hare, 1994; Dreiem and Seegal, 2007; Glaser et al., 2010a,b; Usuki et al., 2008). The inhibition of these complexes or enzymes can contribute to mitochondrial depolarization and swelling up on MeHg exposure. Mitochondrial targeting by MeHg has also been associated with an increase

in mitochondrial overproduction of ROS, which can further exacerbate the toxicity of MeHg by attacking additional nucleophilic centers in mitochondria and in other subcellular compartments (Farina et al., 2011a; Franco et al., 2009; Roos et al., 2011).

Several studies demonstrated that organic and inorganic selenium can influence the deposition of MeHg in the body and protect against its toxicity in animals (Choi et al., 2008; Fredriksson et al., 1993; Ganther, 1971; Glaser et al., 2010b). Selenium (Se) is an essential trace element for a wide range of living organisms, including humans (Allan et al., 1999; Flohe, 1988; Holmgren, 1989; Kumar et al., 1992). Se is necessary for the expression of at least 25 Se-dependent enzymes, including GPx, the TrxR and several other seleno-proteins which modulate the cellular redox and antioxidant status (Bianco et al., 2002; Hatfield and Gladyshev, 2002; Panee et al., 2007).

In addition to inorganic and naturally occurring organoselenium compounds, synthetic organoselenium compounds can also exhibit protective effects against MeHg. Accordingly, ebselen and diphenyl diselenide have been shown to exert beneficial effects against in vitro and in vivo MeHg-induced neurotoxicity (Farina et al., 2003a,b; Moretto et al., 2005a,b; Roos et al., 2009; Usuki et al., 2011; Yin et al., 2011). Diphenyl diselenide (which is the simplest of the diaryl diselenides; Nogueira and Rocha, 2010) protected against some toxic effects of MeHg and lowered the Hg burden in the brain, liver and kidneys of adult mice (de Freitas et al., 2009). The molecular mechanism(s) which underlie(s) the neuroprotective effects of diphenyl diselenide can be related either to the direct interaction of MeHg with “selenol intermediate” of diphenyl diselenide after its reaction with thiols, or indirectly, by the modulation of oxidative stress (via GPx- and/or TrxR-like activities) (de Freitas et al., 2010; Nogueira and Rocha, 2010). In short, the neuroprotective effects of diphenyl diselenide against MeHg-induced toxicity can be related to its antioxidant properties and to its abilities to form stable complexes with MeHg, which can increase Hg excretion and decrease the MeHg body burden. Furthermore, diphenyl diselenide can be partially metabolized to inorganic Se, which may, at least in part, account for its neuroprotective effects against MeHg (Adams et al., 1989).

Therefore, the aim of the present study was to investigate the potential therapeutic effects of DPDS to counteract MeHg-induced toxicity and mitochondrial dysfunction in rats. To accomplish this goal, the effects of DPDS on the deposition of Hg were studied in the brain and liver of rats. An additional goal of this study was to examine the effect of DPDS on the inhibition of TrxR activity in rats exposed to MeHg.

## 2. Materials and Methods

### *2.1. Chemicals*

Chemicals, including ethylene glycol-bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2,4 dinitrophenol (2,4 DNP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), glutamic acid, safranin O, 2',7'-dichlorofluorescin diacetate ($H_2$-DCFDA), and methylmercury chloride (MeHg) were obtained from Sigma Aldrich (St Louis, MO, USA). DPDS was synthesized using the method described by Paulmier (1986). All other chemicals were of analytical reagent grade and purchased from local commercial suppliers.

### *2.2. Animals*

Male Wistar rats, weighing 270–320 g and with age from 3 to 3.5 months from our own breeding colony, were kept in cages of four animals each. They were placed in a room with controlled temperature (22 ± 3°C) on a 12 h light/dark cycle with lights on at 7:00 a.m. and had continuous access to food and water. Our institutional protocols for animal experimentation, designed to minimize suffering and limit the number of animals sacrificed, were followed throughout. All experiments were conducted in accordance with the Committee on Care and Use of Experimental Animal Resources of the Federal University of Santa Maria, Brazil.

### *2.3. Treatment*

Sixteen rats were equally divided into four experimental groups as follows: 1) control (10 mL/Kg of water intragastrically (i.g.) and 1 mL/Kg of soybean oil intraperitoneally (i.p.)); 2) DPDS (10 mL/Kg of water i.g. and 1 mg/Kg of DPDS i.p.); 3) MeHg (5 mg/Kg of MeHg i.g. and 1 mL/Kg of soybean oil i.p.); and 4) DPDS + MeHg (5 mg/Kg of MeHg i.g. and 1 mg/Kg of DPDS i.p.). Exposure protocol was performed daily over 21 days and was based on previous studies (de Freitas et al., 2009; Martins et al., 2009). Twenty-four hours after the last administration, the animals were sacrificed and the livers, brains, kidneys and skeletal muscle were quickly removed from the rats, placed on ice, and homogenized.

### *2.4. Motor coordination tests*

#### *2.4.1. Open field test*

General locomotor activity was evaluated by placing the animals individually in the center of an open-field arena (45 × 45 × 30 cm) made of polywood with black walls and a white floor which was divided into 9 equal squares, as previously described (Broadhurst, 1960). The number of line crossings (number of segments crossed with the four paws) and hearings (expressed by the number of time rearing on the hind limbs) were measured over 5

min and taken as an indicator of locomotor activity. The test was realized 3 times: 24 hours before beginning the treatment (basal), and at the treatment day 11.

*2.4.2. Rotarod test*

Motor coordination skills were tested on the rotarod apparatus as described previously (Lalonde et al., 2003, 2004). Briefly, the rotarod apparatus consists of a rod 30-cm long and 7.5 cm in diameter that rotates at a constant speed of 10 rpm. The rod was flanked by two cardboard plates in order to prevent any escape and suspended at a height of 30 cm. One hour before the first test all animals were trained in the rotarod until they could remain in the apparatus for 60 s without falling. The latency to fall and the number of falls from the apparatus were recorded until 120 s. The tests were conducted 3 times: 24 hours before beginning the treatment (basal), at the treatment day 11, and before the sacrifice.

*2.5. Thioredoxin reductase (TrxR)*

*2.5.1. TrxR purification*

TrxR was partially purified by a modification of the method described by Holmgren and Bjornstedt (1995). Tissues were homogenized in buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4$, and 1.4 mM $KH_2PO_4$, pH 7.3). Livers, brains and kidneys (0.5 g) were homogenized in 10, 3 and 5 volumes of buffered saline, respectively. Homogenates were centrifuged at 13,000 g for 30 min. The protein in the supernatant was measured and adjusted to 10 mg/ml. The supernatant was dialyzed against buffered saline for 16 h to remove endogenous glutathione (GSH) and Trx. The dialyzate was heated at 55°C for 10 min, cooled, and centrifuged at 13,000 g for 30 min to remove denatured protein.

*2.5.2. TrxR activity*

TrxR activity was measured by the method described by Holmgren and Bjornstedt (1995). The reaction mixture consisted of the following: 0.24 mM NADPH, 10 mM EDTA, 100 mM potassium phosphate buffer (pH 7.0), 2 mg/ml 5,5' dithiobis -2-nitrobenzoic acid (DTNB), and 0.2 mg/ml of BSA. The partially purified TrxR was added (to final concentration of 6–8 µg of protein) containing the reaction mixture and the absorbance were followed at 412 nm for a maximum of 4 min.

*2.6. Isolation of rat brain and liver mitochondria*

Rat brain and liver mitochondria were isolated as previously described by Brustovetsky and Dubinsky (2000), with some modifications. Brain and liver were rapidly weighing and homogenized in 1:5 (w/v) ice-cold 'isolation buffer I' containing 225 mM mannitol, 75 mM sucrose, 1 mM $K^+$-EGTA, 0.1% bovine serum albumin (BSA) and 10 mM $K^+$-HEPES, pH 7.2. The tissue was then manually homogenized using a potter glass. The

resulting suspension was centrifuged for 7 min at 2,000 g. After centrifugation the supernatant was re-centrifuged for 10 min at 12,000 g. The pellet was re-suspended in 'isolation buffer II' containing 225 mM mannitol, 75 mM sucrose, 1 mM $K^+$-EGTA, and 10 mM $K^+$-HEPES pH 7.2 and re-centrifuged at 12,000 g for 10 min. The supernatant was discarded and the final pellet gently washed and re-suspended in 'isolation buffer II' without EGTA.

*2.7. Mitochondrial nonprotein and total thiol content*

Mitochondrial nonprotein and total thiol content were measured according to the method of Ellman (1959). To determine total thiol groups, mitochondria (0.3 mg protein) was added to the reaction medium containing 10 mM Tris-HCl pH 7.2, 1% SDS, and 10 mM DTNB. Nonprotein thiol content was measured by adding 50 µL 10% TCA to 50 µL of the mitochondria (0.3 mg protein). After centrifugation (4,000 x g at $4^0$C for 10 min), the protein pellet was discarded, and an aliquot of the clear supernatant, neutralized with 0.1 M NaOH, was added to the medium containing10 mM Tris-HCl pH 7.2, and 10 mM DTNB. The samples absorbance was measured spectrophotometrically at a wavelength of 412 nm.

*2.8. Measurements of mitochondrial transmembrane electrical potential (ΔΨm)*

Mitochondrial ΔΨm was estimated by fluorescence changes in safranin O (3 mM) recorded by RF-5301 Shimadzu spectrofluorometer (Kyoto, Japan) operating at excitation and emission wavelengths of 495 and 535 nm, with 1 slit widths of 1.5 nm (Guo et al., 1998). Mitochondria (0.3 mg protein) were added at 15 seconds of recording to 3 mL standard incubation buffer containing 100 mM sucrose, 65 mM KCl, 10 mM $K^+$-HEPES buffer (pH 7.2), 50 µM EGTA, 200 µM ADP, 400 µM $MgCl_2$, 1 mM Pi, 5mM glutamate and 5 mM succinate. Records were evaluated during 5 minutes. At 250 seconds of recording the 2,4 DNP (100 µM) was added. Data of mitochondrial transmembrane electrical potential (ΔΨm) in Figures are presented as Arbritrary Fluorescence Units (AFU).

*2.9. Estimation of reactive oxygen species (ROS) production*

The mitochondrial generation of ROS was determined spectrofluorimetrically, using the membrane permeable fluorescent dye $H_2$-DCFDA recorded by RF-5301 Shimadzu spectrofluorometer (Kyoto, Japan) operating at excitation and emission wavelengths of 488 and 525 nm, with slit widths of 3 nm (GarciaRuiz et al., 1997). Mitochondria (0.3 mg protein) were added to 3 mL standard incubation buffer containing 100 mM sucrose, 65 mM KCl, 10 mM $K^+$-HEPES buffer (pH 7.2), 50 µM EGTA. Records were evaluated during 5 minutes. At 15 seconds of recording the $H_2$-DCFDA (1 µM) was added. Data of ROS production in figures are presented as Arbritrary Fluorescence Units (AFU).

*2.10. Assessment of mitochondrial metabolic function*

Mitochondria metabolic function was assessed by the conversion of MTT to a dark violet formazan product by mitochondrial dehydrogenases (Mosmann, 1983). MTT (400 µg/ ml) was added to the standard buffer (100 mM sucrose, 65 mM KCl, 10 mM $K^+$-HEPES buffer (pH 7.2), 50 µM EGTA, 200 µM ADP, 400 µM $MgCl_2$, 1 mM Pi, 5mM glutamate and 5 mM succinate) containing an aliquot of mitochondria (0.3 mg protein), and incubated for 45 minutes at $37^0$C. After 45 min the precipitated formazan was solubilized in dimethylsulfoxide (DMSO). The rate of MTT reduction was measured spectrophotometrically at a wavelength of 570 nm. Results were expressed as the percentage of MTT reduction with respect to control values.

*2.11. Assessment of mitochondrial swelling*

Measurement of mitochondrial swelling was performed in a RF-5301 Shimadzu spectrofluorometer at 600 nm (slit 1.5 nm for excitation and emission – Votyakova and Reynolds, 2005). Data for mitochondrial swelling are expressed as Arbitrary Absorbance Units (AAU). The difference (ΔA) between the initial absorbance reading and the final absorbance reading were used for statistical analysis.

*2.12. Determination of Hg levels*

Tissue levels of total Hg were measured in liver, brain and skeletal muscle collected at the time of euthanasia. Approximately 0.4 g of the tissues were weighed and digested with 5 mL of $HNO_3$ acid (65%). Digested samples were diluted to 50 mL with ultrapure water before analysis by Inductively Coupled Plasma Mass Spectroscopy (ICP-MS).

*2.13. Protein measurement*

Protein was assayed by the method of Bradford (1976) with bovine serum albumin as standard.

*2.14. Statistical analysis*

Data were analyzed statistically by one- or two-way analysis of variance (ANOVA), followed by Duncan's post-hoc tests when appropriate. The results were considered statistically significant when $p<0.05$.

## 3. Results

*3.1. Effects of DPDS and MeHg on body weight.*

Treatment with MeHg induced a loss of body weight from the second week until the end of the treatment when compared to the control rats ($p < 0.05$, Figure 1). Rats that receveid the co-treatment with DPDS and MeHg also showed a decrease in the body weight when compared to control and DPDS groups ($p < 0.05$, Figure 1). Rats treated with DPDS lost

weight after the first week of treatment ($p < 0.05$), but they gradually recover their weights being statistically undistinguishable from the controls at the end of the treatment (Figure 1).

*3.2. Effects of DPDS and MeHg on motor coordination and spontaneous locomotor activity*

The effects of the treatments with MeHg and/ or DPDS on the locomotion and motor coordination were verified by the open-field and rotarod tests, respectively. Rats treated with MeHg after 11 days presented an increase in the number of falls on the rotarod and a decrease in the latency to the first fall when compared to control rats ($p < 0.05$, Figure 2A, 2B). Rats treated with DPDS did not present statistical differences on the rotarod test when compared to control rats; however, rats co-treated with DPDS and MeHg presented an increase in loss of motor coordination observed by an increased number of falls and reduced latency to the first fall when compared to the other groups ($p < 0.05$, Figure 2A, 2B). The rotarod test could not be performed at the end of the treatment in those rats receiving MeHg since they were incapable to stay at the apparatus due to serious motor impairment caused by MeHg.

Rats treated with MeHg showed a decrease in the number of crossings on the open-field at the end of the treatment when compared to the control rats ($p < 0.05$, Figure 3A). Rats that received the co-treatment with MeHg and DPDS also presented a significant decrease in the number of crossings (from the treatment days 11), and a decrease in the number of rearings (at the end of the treatment) when compared to the control rats ($p < 0.05$, Figure 3B). Treatment with DPDS did not interfere in the performance of the rats on the open-field. The decrease in the number of crossings and rearings observed in all groups at treatment days 11 and 21 when compared to the basal is expected since the animals stay familiarized with the open-field arena.

*3.3. Effects of DPDS and MeHg on TrxR activity*

MeHg has been demonstrated to preferentially inhibit TrxR activity both *in vitro* and *in vivo* (Carvalho et al., 2008; Carvalho et al., 2010; Branco et al., 2011). Since DPDS was shown to be a substrate for TrxR, we decided to measure the activity of this enzyme in different tissues of rats treated with MeHg and/ or DPDS. Rats treated with DPDS presented hepatic and renal TrxR activities increased when compared to the controls rats, while the cerebral TrxR activity presented only a tendency to increase ($p < 0.05$, Figure 4). Treatment with MeHg caused a decrease in the activity of TrxR from liver, kidney and brain when

compared to the control rats ($p<0.05$, Figure 4). Co-treatment with DPDS could not restore MeHg-inhibited TrxR activity in the liver, kidney and brain of rats (Figure 4).

*3.4. Effects of DPDS and MeHg on mitochondrial dysfunction*

*3.4.1. Mitochondrial metabolic function*

The hepatic mitochondrial metabolic function (MTT reduction) was not affected by any treatment (Figure 5). In brain, treatment with MeHg or co-treatment with MeHg and DPDS decreased the capacity of mitochondrial dehydrogenases to reduce MTT when compared to mitochondria from control rats ($p < 0.05$, Figure 5). Treatment with DPDS alone did not affect the cerebral mitochondrial metabolic function of rats.

*3.4.2. Mitochondrial total and nonprotein thiols*

MeHg treatment caused a decreased in the mitochondrial total thiol levels in brain and liver when compared to control rats ($p < 0.05$, Figure 6). Treatment with DPDS alone did not alter the mitochondrial total thiol levels from liver and brain of rats (Figure 6). The co-treatment with DPDS recovered MeHg-induced mitochondrial total thiol levels depletion in rat's liver and brain ($p < 0.05$, Figure 6). Rats treated with MeHg presented decreased levels of mitochondrial nonprotein thiols in liver when compared to control rats and the co-administration with DPDS recovered the nonprotein thiol contents to control levels ($p < 0.05$, Figure 7A). Mitochondrial nonprotein thiols levels were not affected by any treatment in brain (Figure 7B).

*3.4.3. Mitochondrial swelling*

Treatment with MeHg statistically increased the mitochondrial swelling in liver when compared to control rats ($p < 0.05$, Figure 8A). The co-treatment with DPDS partially prevented MeHg-induced mitochondrial swelling in liver, but these values were not statistically different from the values found for rats exposed only to MeHg (Figure 8A). Treatment with MeHg also increased the mitochondrial swelling in brain ($p < 0.05$) and the co-treatment with DPDS did not protect from this increase in mitochondria swelling induced by MeHg ($p < 0.05$, Figure 8B). Treatment with DPDS alone did not alter the mitochondrial swelling in brain and liver of rats when compared to the control group (Figure A and B)

*3.4.4. Mitochondrial ROS production*

Mitochondrial ROS production (DCFH oxidation) was significantly increased in liver of rats treated with MeHg or co-treated with MeHg and DPDS ($p < 0.05$, Figure 9A). Rats treated with DPDS presented hepatic mitochondrial ROS levels similar to the control. ROS production by mitochondria of brain was not affected by any treatment (Figure 9B).

*3.4.5. Mitochondrial ΔΨm*

Polarization (ΔΨm) of mitochondria from liver of rats co-treated with MeHg and DPDS was significantly reduced when compared to the other groups ($p < 0.05$, Figure 10A). Treatment with DPDS and MeHg alone did not cause mitochondrial depolarization in liver of rats. Treatment with DPDS and/ or MeHg had no effect in mitochondrial ΔΨm in brain of rats (Figure 10B).

*3.5. Effects of DPDS and MeHg on Hg content*

Treatment with MeHg increased the levels of Hg in brain, liver and skeletal muscle of rats when compared to the rats not exposed to MeHg ($p < 0.05$, Figure 11). The co-treatment with DPDS caused an additional increase in the deposition of Hg when compared to MeHg alone in brain and liver, and had a tendency to increase in the skeletal muscle (Figure 11). Two-way ANOVA of mercury levels revealed a significant interaction between MeHg and DPDS both in brain and in liver ($F_{1,16}=13.310$, $F_{1,16}=5.537$, respectively, $p < 0.05$).

**Discussion**

The present study investigated the potential protective effect of DPDS, an organoselenium compound, in attenuate MeHg-induced toxicity in rats. The results herein showed that the co-treatment with DPDS and MeHg increased Hg deposition in the brain and liver of exposed rats. These results differ from those of de Freitas et al. (2009) where DPDS led to a significant reduction in Hg concentrations in brain, liver and kidney of MeHg-exposed mice. The discrepancies between the 2 works could be attributed to the differences between the species and the route of administration, since in the present work it was used rats and the via of administration for DPDS was i.p., and in the study of de Freitas (2009) it was used mice and DPDS was administered subcutaneously (s.c.). In the study of de Freitas et al. (2009) the proposed mechanism for the reduction in organs Hg burden by diphenyl diselenide is the formation of a selenol/selenolate (PhSeH/PhSe−) intermediate which could interact with MeHg, generating the readily excretable PhSeHgMe complex. One possible explanation for the increase in hepatic and cerebral Hg deposition by the co-treatment with DPDS observed in this study could be the conversion of DPDS to selenhidric acid, this selenium compound could bind to MeHg to form a less soluble complex (Pinheiro et al., 2009). Thus, the increase in hepatic and cerebral Hg deposition by the co-treatment with DPDS possibly involves Hg:Se interactions but with the formation of a less excretable compound that can accumulate in critical organs (Pinheiro et al., 2009). These results are in agreement with other studies that

showed an elevated deposition of Hg in key brain regions after oral selenium administration (Moller-Madsen and Danscher, 1991; Newland et al., 2006; Prohaska and Ganther, 1977; Schionning, 2000; Schionning et al., 1997).

The decreased weight gain or even loss of weight is a prominent and readily observed sign of severe MeHg toxicity in experimental animals. In this study, rats treated with MeHg presented body weight lost, although the most severe loss of body weight occurred in rats co-treated with DPDS and MeHg. In addition, rats treated with MeHg showed a decrease in locomotor activity observed by the rotarod and open-field tests. The co-treatment with DPDS and MeHg also increased the loss of motor coordination (rotarod test) in rats. These results indicate that the co-treatment with DPDS increased the neurotoxic effects of MeHg in rats. The motor deficits are generally the most evident neurological effects following MeHg exposure (Dietrich et al., 2005; Goulet et al., 2003; Kim et al., 2000; Rice, 1996). In vivo studies based mainly on the use of rodents, point to impairment in intracellular calcium homeostasis, alteration of glutamate homeostasis and oxidative stress as important events in MeHg-induced neurotoxicity. The overactivation of NMDA-type glutamate receptors increases $Ca^{2+}$ influx into neurons, therefore leading to the activation of important pathways involved with cell death (Choi, 1992; Pivovarova and Andrews, 2010). Alternatively, $Ca^{2+}$ can be taken up by mitochondria, where it may stimulate the generation of reactive oxygen species (ROS) (Ceccatelli et al., 2010; Lafon-Cazal et al., 1993; Reynolds and Hastings, 1995).

Consistent with this hypothesis, several studies corroborates MeHg's ability to induce mitochondrial dysfunction and consequent generation of ROS (Mori et al., 2007). In view of this, we investigated MeHg toxicity to brain and liver mitochondria of exposed rats. The results present herein showed that exposure to MeHg induced mitochondrial dysfunction observed by the increase in mitochondrial swelling and decrease in mitochondrial metabolic function (mitochondrial dehydrogenases activity) in brain, and the increase in mitochondrial swelling and ROS production (DCFH oxidation) in liver. The high affinity of MeHg for sulfhydryl (-SH) groups is considered an important mechanism in mediating MeHg toxicity since the binding of MeHg to –SH groups inactivates enzymes including respiratory chain complexes (Farina et al., 2011a,b; Glaser et al., 2010a,b; Mori et al., 2007; Valko et al., 2005). Consistent with this, is the decrease in mitochondrial dehydrogenases activity in brain of exposed rats. In the same way, the inhibition of these complexes can contribute to mitochondrial swelling and ROS production on MeHg exposure. Alternatively, the co-treatment with DPDS prevented mitochondrial nonprotein and total thiol groups depletion

induced by MeHg in brain and liver of rats. Accordingly, the DPDS-prevented thiol depletion may reside in its ability to form a complex with MeHg thus effectively reducing MeHg binding to protein and free thiols. However, the co-treatment with DPDS did not protect from the MeHg-induced mitochondrial swelling and decrease in mitochondrial metabolic function in brain and increase in mitochondrial ROS production in liver. These indicate that, mechanisms other than the interaction with important free- and protein-thiols, may be involved in the MeHg-induced mitochondrial dysfunction observed here.

Previous studies have demonstrated that MeHg can directly inhibit TrxR activity both *in vitro* and *in vivo* (Branco et al., 2012; Carvalho et al., 2011; Wagner et al., 2010a). Here, we show that MeHg treatment inhibited TrxR activity from brain, liver and kidney of rats. MeHg forms covalent bounds between its Hg moiety and the selenium of the selenocysteine of the enzyme, thus directly inhibiting the activity of TrxR (Carvalho et al., 2011). Since TrxR is critical for the cellular antioxidant defense system the inhibition of this enzyme could have a central role in the toxicity of MeHg. Recently, diphenyl diselenide was demonstrated to be a substrate for cerebral and hepatic rat TrxR which could account at least in part for the antioxidant properties of DPDS (de Freitas et al., 2010; de Freitas and Rocha, 2011). In our study, rats treated solely with DPDS showed an increase in the activity of renal and hepatic TrxR. The formation of selenhidric acid from DPDS could also explain the increase in TrxR activity, since this inorganic form of selenium could be converted to selenocystein and incorporated to selenoenzymes such as TrxR (Pinheiro et al., 2009). The co-treatment with DPDS and MeHg was not able to avoid or recover MeHg-inhibited TrxR activity of brain, liver and kidney of rats. Similarly, studies *in vitro* and *in vivo* showed that selenite was capable of recover the activity of TrxR when inhibited by $HgCl_2$ but not by MeHg, and the effect of selenite was attributed to the displacement of the Hg from the active site, given rise to mercury selenide and regenerating the TrxR selenol (Branco et al., 2012; Carvalho et al., 2011).

In conclusion, the results of this study showed that DPDS, at a low dose, can increase Hg body burden and MeHg neurotoxicity in rats. The results present here also reinforce the central role of the mitochondrial dysfunction on the toxicity induced by MeHg exposure *in vivo* as well as the role of TrxR as a molecular target for MeHg in rats. Further research into MeHg-DPDS interactions will help to better understand the consequences of the concomitantly exposure to these compounds.

**Acknowledgments**

Supported by FAPERGS (Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), FINEP (Rede Instituto Brasileiro de Neurociência (IBN-Net) # 01.06.0842-00), FAPERGS-PRONEX-CNPQ and INCT-EN (Instituto Nacional de Ciência e Tecnologia em Excitotoxicidade e Neuroproteção).

**References**

Adams, W.J., Jr., Kocsis, J.J., Snyder, R., 1989. Acute toxicity and urinary excretion of diphenyldiselenide. Toxicol Lett. 48**,** 301-10.

Allan, C.B., Lacourciere, G.M., Stadtman, T.C., 1999. Responsiveness of selenoproteins to dietary selenium. Annu Rev Nutr. 19**,** 1-16.

Arner, E.S.J., Holmgren, A., 2000. Physiological functions of thioredoxin and thioredoxin reductase. European Journal of Biochemistry. 267**,** 6102-6109.

Aschner, M., Onishchenko, N., Ceccatelli, S., 2010. Toxicology of alkylmercury compounds. Met Ions Life Sci. 7**,** 403-34.

Atchison, W.D., Hare, M.F., 1994. Mechanisms of methylmercury-induced neurotoxicity. FASEB J. 8**,** 622-9.

Bianco, A.C., Salvatore, D., Gereben, B., Berry, M.J., Larsen, P.R., 2002. Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. Endocr Rev. 23**,** 38-89.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72**,** 248-54.

Branco, V., Canario, J., Holmgren, A., Carvalho, C., 2011. Inhibition of the thioredoxin system in the brain and liver of zebra-seabreams exposed to waterborne methylmercury. Toxicol Appl Pharmacol. 251**,** 95-103.

Broadhurst, P.L., 1960. The place of animal psychology in the development of psychosomatic research. Fortschr Psychosom Med. 1**,** 63-9.

Brustovetsky, N., Dubinsky, J.M., 2000. Dual responses of CNS mitochondria to elevated calcium. J Neurosci. 20**,** 103-13.

Carvalho, C.M., Matos, A.I., Mateus, M.L., Santos, A.P., Batoreu, M.C., 2008. High-fish consumption and risk prevention: assessment of exposure to methylmercury in Portugal. J Toxicol Environ Health A. 71**,** 1279-88.

Carvalho, C.M., Lu, J., Zhang, X., Arner, E.S., Holmgren, A., 2011. Effects of selenite and chelating agents on mammalian thioredoxin reductase inhibited by mercury: implications for treatment of mercury poisoning. FASEB J. 25**,** 370-81.

Ceccatelli, S., Dare, E., Moors, M., 2010. Methylmercury-induced neurotoxicity and apoptosis. Chem Biol Interact. 188**,** 301-8.

Choi, A.L., Budtz-Jorgensen, E., Jorgensen, P.J., Steuerwald, U., Debes, F., Weihe, P., Grandjean, P., 2008. Selenium as a potential protective factor against mercury developmental neurotoxicity. Environmental Research. 107**,** 45-52.

Choi, D.W., 1992. Excitotoxic cell death. J Neurobiol. 23**,** 1261-76.

Clarkson, T.W., Magos, L., Myers, G.J., 2003. The toxicology of mercury - Current exposures and clinical manifestations. New England Journal of Medicine. 349**,** 1731-1737.

Compeau, G.C., Bartha, R., 1985. Sulfate-reducing bacteria: principal methylators of mercury in anoxic estuarine sediment. Appl Environ Microbiol. 50**,** 498-502.

Costa, L.G., Aschner, M., Vitalone, A., Syversen, T., Soldin, O.P., 2004. Developmental neuropathology of environmental agents. Annu Rev Pharmacol Toxicol. 44**,** 87-110.

de Freitas, A.S., Funck, V.R., Rotta, M.D., Bohrer, D., Morschbacher, V., Puntel, R.L., Nogueira, C.W., Farina, M., Aschner, M., Rocha, J.B.T., 2009. Diphenyl diselenide, a simple organoselenium compound, decreases methylmercury-induced cerebral, hepatic and renal oxidative stress and mercury deposition in adult mice. Brain Research Bulletin. 79**,** 77-84.

de Freitas, A.S., Prestes, A.D., Wagner, C., Sudati, J.H., Alves, D., Porciuncula, L.O., Kade, I.J., Rocha, J.B.T., 2010. Reduction of Diphenyl Diselenide and Analogs by Mammalian Thioredoxin Reductase Is Independent of Their Gluthathione Peroxidase-Like Activity: A Possible Novel Pathway for Their Antioxidant Activity. Molecules. 15**,** 7699-7714.

de Freitas, A.S., Rocha, J.B., 2011. Diphenyl diselenide and analogs are substrates of cerebral rat thioredoxin reductase: a pathway for their neuroprotective effects. Neurosci Lett. 503**,** 1-5.

Dietrich, M.O., Mantese, C.E., Anjos, G.D., Souza, D.O., Farina, M., 2005. Motor impairment induced by oral exposure to methylmercury in adult mice. Environ Toxicol Pharmacol. 19**,** 169-75.

Dorea, J.G., 2008. Persistent, bioaccumulative and toxic substances in fish: human health considerations. Sci Total Environ. 400**,** 93-114.

Dreiem, A., Seegal, R.F., 2007. Methylmercury-induced changes in mitochondrial function in striatal synaptosomes are calcium-dependent and ROS-independent. Neurotoxicology. 28**,** 720-726.

Ellman, G.L., 1959. Tissue sulfhydryl groups. Arch Biochem Biophys. 82**,** 70-7.

Farina, M., Dahm, K.C., Schwalm, F.D., Brusque, A.M., Frizzo, M.E., Zeni, G., Souza, D.O., Rocha, J.B., 2003a. Methylmercury increases glutamate release from brain synaptosomes and glutamate uptake by cortical slices from suckling rat pups: modulatory effect of ebselen. Toxicol Sci. 73**,** 135-40.

Farina, M., Frizzo, M.E., Soares, F.A., Schwalm, F.D., Dietrich, M.O., Zeni, G., Rocha, J.B., Souza, D.O., 2003b. Ebselen protects against methylmercury-induced inhibition of glutamate uptake by cortical slices from adult mice. Toxicol Lett. 144**,** 351-7.

Farina, M., Campos, F., Vendrell, I., Berenguer, J., Barzi, M., Pons, S., Sunol, C., 2009. Probucol increases glutathione peroxidase-1 activity and displays long-lasting protection against methylmercury toxicity in cerebellar granule cells. Toxicol Sci. 112**,** 416-26.

Farina, M., Aschner, M., Rocha, J.B., 2011a. Oxidative stress in MeHg-induced neurotoxicity. Toxicol Appl Pharmacol.

Farina, M., Rocha, J.B., Aschner, M., 2011b. Mechanisms of methylmercury-induced neurotoxicity: Evidence from experimental studies. Life Sci.

Flohe, L., Gunzler, W.A., Schock, H.H., 1973. Glutathione peroxidase: a selenoenzyme. FEBS Lett. 32**,** 132-4.

Flohe, L., 1988. Glutathione peroxidase. Basic Life Sci. 49**,** 663-8.

Franco, J.L., Braga, H.C., Stringari, J., Missau, F.C., Posser, T., Mendes, B.G., Leal, R.B., Santos, A.R., Dafre, A.L., Pizzolatti, M.G., Farina, M., 2007. Mercurial-induced hydrogen peroxide generation in mouse brain mitochondria: protective effects of quercetin. Chem Res Toxicol. 20**,** 1919-26.

Franco, J.L., Posser, T., Dunkley, P.R., Dickson, P.W., Mattos, J.J., Martins, R., Bainy, A.C.D., Marques, M.R., Dafre, A.L., Farina, M., 2009. Methylmercury neurotoxicity is associated with inhibition of the antioxidant enzyme glutathione peroxidase. Free Radical Biology and Medicine. 47**,** 449-457.

Fredriksson, A., Gardlund, A.T., Bergman, K., Oskarsson, A., Ohlin, B., Danielsson, B., Archer, T., 1993. Effects of Maternal Dietary Supplementation with Selenite on the Postnatal-Development of Rat Offspring Exposed to Methyl Mercury in-Utero. Pharmacology & Toxicology. 72**,** 377-382.

Ganther, H.E., 1971. Reduction of Selenotrisulfide Derivative of Glutathione to a Persulfide Analog by Glutathione Reductase. Biochemistry. 10**,** 4089-&.

GarciaRuiz, C., Colell, A., Mari, M., Morales, A., FernandezCheca, J.C., 1997. Direct effect of ceramide on the mitochondrial electron transport chain leads to generation of

reactive oxygen species - Role of mitochondrial glutathione. Journal of Biological Chemistry. 272**,** 11369-11377.

Glaser, V., Leipnitz, G., Straliotto, M.R., Oliveira, J., dos Santos, V.V., Wannmacher, C.M.D., de Bern, A.F., Rocha, J.B.T., Farina, M., Latini, A., 2010a. Oxidative stress-mediated inhibition of brain creatine kinase activity by methylmercury. Neurotoxicology. 31**,** 454-460.

Glaser, V., Nazari, E.M., Muller, Y.M., Feksa, L., Wannmacher, C.M., Rocha, J.B., de Bem, A.F., Farina, M., Latini, A., 2010b. Effects of inorganic selenium administration in methylmercury-induced neurotoxicity in mouse cerebral cortex. Int J Dev Neurosci. 28**,** 631-7.

Goulet, S., Dore, F.Y., Mirault, M.E., 2003. Neurobehavioral changes in mice chronically exposed to methylmercury during fetal and early postnatal development. Neurotoxicol Teratol. 25**,** 335-47.

Grandjean, P., Herz, K.T., 2011. Methylmercury and brain development: imprecision and underestimation of developmental neurotoxicity in humans. Mt Sinai J Med. 78**,** 107-18.

Guo, Q., Christakos, S., Robinson, N., Mattson, M.P., 1998. Calbindin D28k blocks the proapoptotic actions of mutant presenilin 1: reduced oxidative stress and preserved mitochondrial function. Proc Natl Acad Sci U S A. 95**,** 3227-32.

Hassan, S.A., Moussa, E.A., Abbott, L.C., 2011. The effect of methylmercury exposure on early central nervous system development in the zebrafish (Danio rerio) embryo. J Appl Toxicol.

Hatfield, D.L., Gladyshev, V.N., 2002. How selenium has altered our understanding of the genetic code. Mol. Cell Biol. 22, 3565-3576.

Holmgren, A., 1989. Thioredoxin and glutaredoxin systems. J Biol Chem. 264**,** 13963-6.

Holmgren, A., Bjornstedt, M., 1995. Thioredoxin and thioredoxin reductase. Methods Enzymol. 252**,** 199-208.

Johansson, C., Castoldi, A.F., Onishchenko, N., Manzo, L., Vahter, M., Ceccatelli, S., 2007. Neurobehavioural and molecular changes induced by methylmercury exposure during development. Neurotox Res. 11**,** 241-60.

Kaur, P., Aschner, M., Syversen, T., 2006. Glutathione modulation influences methyl mercury induced neurotoxicity in primary cell cultures of neurons and astrocytes. Neurotoxicology. 27**,** 492-500.

Kim, C.Y., Nakai, K., Kasanuma, Y., Satoh, H., 2000. Comparison of neurobehavioral changes in three inbred strains of mice prenatally exposed to methylmercury. Neurotoxicol. Teratol. 22, 397–403.

Kumar, S., Bjornstedt, M., Holmgren, A., 1992. Selenite is a substrate for calf thymus thioredoxin reductase and thioredoxin and elicits a large non-stoichiometric oxidation of NADPH in the presence of oxygen. Eur J Biochem. 207**,** 435-39.

Kuntz, S.W., Hill, W.G., Linkenbach, J.W., Lande, G., Larsson, L., 2009. Methylmercury risk and awareness among American Indian women of childbearing age living on an inland northwest reservation. Environmental Research. 109**,** 753-759.

Lafon-Cazal, M., Pietri, S., Culcasi, M., Bockaert, J., 1993. NMDA-dependent superoxide production and neurotoxicity. Nature. 364**,** 535-7.

Lalonde, R., Lewis, T.L., Strazielle, C., Kim, H., Fukuchi, K., 2003. Transgenic mice expressing the betaAPP695SWE mutation: effects on exploratory activity, anxiety, and motor coordination. Brain Res. 977**,** 38-45.

Lalonde, R., Kim, H.D., Fukuchi, K., 2004. Exploratory activity, anxiety, and motor coordination in bigenic APPswe + PS1/DeltaE9 mice. Neurosci Lett. 369**,** 156-61.

Lillig, C.H., Holmgren, A., 2007. Thioredoxin and related molecules - From biology to health and disease. Antioxidants & Redox Signaling. 9**,** 25-47.

Marsh, D.O., Clarkson, T.W., Myers, G.J., Davidson, P.W., Cox, C., Cernichiari, E., Tanner, M.A., Lednar, W., Shamlaye, C., Choisy, O., et al., 1995. The Seychelles study of fetal methylmercury exposure and child development: introduction. Neurotoxicology. 16**,** 583-96.

Moller-Madsen, B., Danscher, G., 1991. Localization of mercury in CNS of the rat. IV. The effect of selenium on orally administered organic and inorganic mercury. Toxicol Appl Pharmacol. 108**,** 457-73.

Moretto, M.B., Funchal, C., Santos, A.Q., Gottfried, C., Boff, B., Zeni, G., Pureur, R.P., Souza, D.O., Wofchuk, S., Rocha, J.B., 2005a. Ebselen protects glutamate uptake inhibition caused by methyl mercury but does not by Hg2+. Toxicology. 214**,** 57-66.

Moretto, M.B., Funchal, C., Zeni, G., Pessoa-Pureur, R., Rocha, J.B., 2005b. Selenium compounds prevent the effects of methylmercury on the in vitro phosphorylation of cytoskeletal proteins in cerebral cortex of young rats. Toxicol Sci. 85**,** 639-46.

Mori, N., Yasutake, A., Hirayama, K., 2007. Comparative study of activities in reactive oxygen species production/defense system in mitochondria of rat brain and liver, and their susceptibility to methylmercury toxicity. Arch Toxicol. 81**,** 769-76.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 65**,** 55-63.

Newland, M.C., Reed, M.N., LeBlanc, A., Donlin, W.D., 2006. Brain and blood mercury and selenium after chronic and developmental exposure to methylmercury. Neurotoxicology. 27, 710-720.

Nogueira, C.W., Rocha, J.B.T., 2010. Diphenyl Diselenide a Janus-Faced Molecule. Journal of the Brazilian Chemical Society. 21**,** 2055-2071.

Panee, J., Stoytcheva, Z.R., Liu, W., Berry, M.J., 2007. Selenoprotein H is redox-sensing high mobility group family DNA-binding protein that up-regulates genes involved in glutathione synthesis and phase II detoxification. Journal of Biological Chemistry. 282**,** 23759-23765.

Paulmier, C., 1986. Selenoorganic functional groups. In Selenium reagents and intermediates in organic synthesis., Vol., ed.^eds. Pergamon Press, Oxford, pp. 25-51.

Pinheiro, M.C., Do Nascimento, J.L.M., Silveira, L.C.L., Da Rocha, J.B.T., Aschner, M., 2009. Mercury and Selenium – A Review on Aspects Related to the Health of Human Populations in the Amazon. 4, 222–245.

Pivovarova, N.B., Andrews, S.B., 2010. Calcium-dependent mitochondrial function and dysfunction in neurons. FEBS J. 277**,** 3622-36.

Prohaska, J.R., Ganther, H.E., 1977. Interactions between selenium and methylmercury in rat brain. Chem Biol Interact. 16**,** 155-67.

Reynolds, I.J., Hastings, T.G., 1995. Glutamate induces the production of reactive oxygen species in cultured forebrain neurons following NMDA receptor activation. J Neurosci. 15**,** 3318-27.

Rice, D.C., 1996. Evidence for delayed neurotoxicity produced by methylmercury. Neurotoxicology. 17**,** 583-96.

Roos, D.H., Puntel, R.L., Santos, M.M., Souza, D.O., Farina, M., Nogueira, C.W., Aschner, M., Burger, M.E., Barbosa, N.B., Rocha, J.B., 2009. Guanosine and synthetic organoselenium compounds modulate methylmercury-induced oxidative stress in rat brain cortical slices: involvement of oxidative stress and glutamatergic system. Toxicol In Vitro. 23**,** 302-7.

Roos, D.H., Puntel, R.L., Farina, M., Aschner, M., Bohrer, D., Rocha, J.B., de Vargas Barbosa, N.B., 2011. Modulation of methylmercury uptake by methionine: prevention of mitochondrial dysfunction in rat liver slices by a mimicry mechanism. Toxicol Appl Pharmacol. 252**,** 28-35.

Sandalova, T., Zhong, L., Lindqvist, Y., Holmgren, A., Schneider, G., 2001. Three-dimensional structure of a mammalian thioredoxin reductase: implications for mechanism and evolution of a selenocysteine-dependent enzyme. Proc Natl Acad Sci U S A. 98**,** 9533-8.

Schionning, J.D., 2000. Experimental neurotoxicity of mercury. Autometallographic and stereologic studies on rat dorsal root ganglion and spinal cord. APMIS Suppl. 99, 1-32.

Schionning, J.D., Eide, R., Ernst, E., Danscher, G., Moller-Madsen, B., 1997. The effect of selenium on the localization of autometallographic mercury in dorsal root ganglia of rats. Histochem J. 29, 183-191.

Shanker, G., Aschner, J.L., Syversen, T., Aschner, M., 2004. Free radical formation in cerebral cortical astrocytes in culture induced by methylmercury. Brain Res Mol Brain Res. 128**,** 48-57.

Skerfving, S., 1978. Interaction between selenium and methylmercury. Environ Health Perspect. 25**,** 57-65.

Sugiura, Y., Hojo, Y., Tamai, Y., Tanaka, H., 1976. Letter: Selenium protection against mercury toxicity. Binding of methylmercury by the selenohydryl-containing ligand. J Am Chem Soc. 98**,** 2339-41.

Usuki, F., Fujita, E., Sasagawa, N., 2008. Methylmercury activates ASK1/JNK signaling pathways, leading to apoptosis due to both mitochondria- and endoplasmic reticulum (ER)-generated processes in myogenic cell lines. Neurotoxicology. 29**,** 22-30.

Usuki, F., Yamashita, A., Fujimura, M., 2011. Post-transcriptional defects of antioxidant selenoenzymes cause oxidative stress under methylmercury exposure. J Biol Chem. 286**,** 6641-9.

Valko, M., Morris, H., Cronin, M.T., 2005. Metals, toxicity and oxidative stress. Curr Med Chem. 12**,** 1161-208.

Votyakova, T.V., Reynolds, I.J., 2005. Ca2+-induced permeabilization promotes free radical release from rat brain mitochondria with partially inhibited complex I. J Neurochem. 93**,** 526-37.

Wagner, C., Sudati, J.H., Nogueira, C.W., Rocha, J.B., 2010a. In vivo and in vitro inhibition of mice thioredoxin reductase by methylmercury. Biometals. 23**,** 1171-7.

Wagner, C., Vargas, A.P., Roos, D.H., Morel, A.F., Farina, M., Nogueira, C.W., Aschner, M., Rocha, J.B., 2010b. Comparative study of quercetin and its two glycoside derivatives

quercitrin and rutin against methylmercury (MeHg)-induced ROS production in rat brain slices. Arch Toxicol. 84**,** 89-97.

Yin, Z., Lee, E., Ni, M., Jiang, H., Milatovic, D., Rongzhu, L., Farina, M., Rocha, J.B., Aschner, M., 2011. Methylmercury-induced alterations in astrocyte functions are attenuated by ebselen. Neurotoxicology. 32**,** 291-9.

Zhong, L.W., Arner, E.S.J., Holmgren, A., 2000. Structure and mechanism of mammalian thioredoxin reductase: The active site is a redox-active selenolthiol/selenenylsulfide formed from the conserved cysteine-selenocysteine sequence. Proceedings of the National Academy of Sciences of the United States of America. 97**,** 5854-5859.

**Legends**

**Figure 1. Effect of methylmercury (MeHg) and/ or diphenyl diselenide (DPDS) on the body weight.** Data are expressed as mean ± S.E.M., n=4. (*) represents $p<0.05$ as compared to control by Duncan's multiple range test. (#) represents $p<0.05$ as compared to DPDS by Duncan's multiple range test.

**Figure 2. Rotarod test in rats exposed to methylmercury (MeHg) and/ or diphenyl diselenide (DPDS). The number of falls (A) and latency for the first fall (B) were recorded.** Data are expressed as mean ± S.E.M., n=4. Bars with different letters are significantly different ($P<0.05$) from each other (one-way ANOVA followed by the Duncan multiple range test).

**Figure 3. Open field test in rats exposed to methylmercury (MeHg) and/ or diphenyl diselenide (DPDS). Ambulation (crossing) (A) and rearing (B) were recorded.** Data are expressed as mean ± S.E.M., n=4. Bars with different letters are significantly different ($P<0.05$) from each other (one-way ANOVA followed by the Duncan multiple range test).

**Figure 4. Thioredoxin reductase activity in liver (A), kidney (B) and brain (C) of rats exposed to methylmercury (MeHg) and/ or diphenyl diselenide (DPDS).** Data are expressed as mean ± S.E.M., n=4. Bars with different letters are significantly different ($P<0.05$) from each other (one-way ANOVA followed by the Duncan multiple range test).

**Figure 5. MTT reduction in liver (A) and brain (B) mitochondria of rats exposed to methylmercury (MeHg) and/ or diphenyl diselenide (DPDS).** Data are expressed as mean ± S.E.M., n=4. Bars with different letters are significantly different ($P<0.05$) from each other (one-way ANOVA followed by the Duncan multiple range test).

**Figure 6. Total thiol content in liver (A) and brain (B) mitochondria of rats exposed to methylmercury (MeHg) and/ or diphenyl diselenide (DPDS).** Data are expressed as mean ± S.E.M., n=4. Bars with different letters are significantly different ($P<0.05$) from each other (one-way ANOVA followed by the Duncan multiple range test).

**Figure 7. Nonprotein thiol content in liver (A) and brain (B) mitochondria of rats exposed to methylmercury (MeHg) and/ or diphenyl diselenide (DPDS).** Data are expressed as mean ± S.E.M., n=4. Bars with different letters are significantly different ($P<0.05$) from each other (one-way ANOVA followed by the Duncan multiple range test).

**Figure 8. Mitochondrial swelling in liver (A) and brain (B) of rats exposed to methylmercury (MeHg) and/ or diphenyl diselenide (DPDS).** Data are expressed as mean

± S.E.M., n=4. Bars with different letters are significantly different (P<0.05) from each other (one-way ANOVA followed by the Duncan multiple range test).

**Figure 9. ROS production ($H_2$-DCFH oxidation) in liver (A) and brain (B) mitochondria of rats exposed to methylmercury (MeHg) and/ or diphenyl diselenide (DPDS). Insets represent statistical analysis of the 300 s timepoint.** Data are expressed as mean ± S.E.M., n=4. Bars and lines with different letters are significantly different (P<0.05) from each other (one-way ANOVA followed by the Duncan multiple range test).

**Figure 10. Mitochondrial depolarization in liver (A) and brain (B) of rats exposed to methylmercury (MeHg) and/ or diphenyl diselenide (DPDS).** Data are expressed as mean ± S.E.M., n=4. (*) represents p<0.05 as compared to control by Duncan's multiple range test.

**Figure 11. Hg content in liver (A), brain (B), and muscle (C) of rats exposed to methylmercury (MeHg) and/ or diphenyl diselenide (DPDS).** Data are expressed as mean ± S.E.M., n=4. Bars with different letters are significantly different (P<0.05) from each other (one-way ANOVA followed by the Duncan multiple range test).

**Figure 1.**

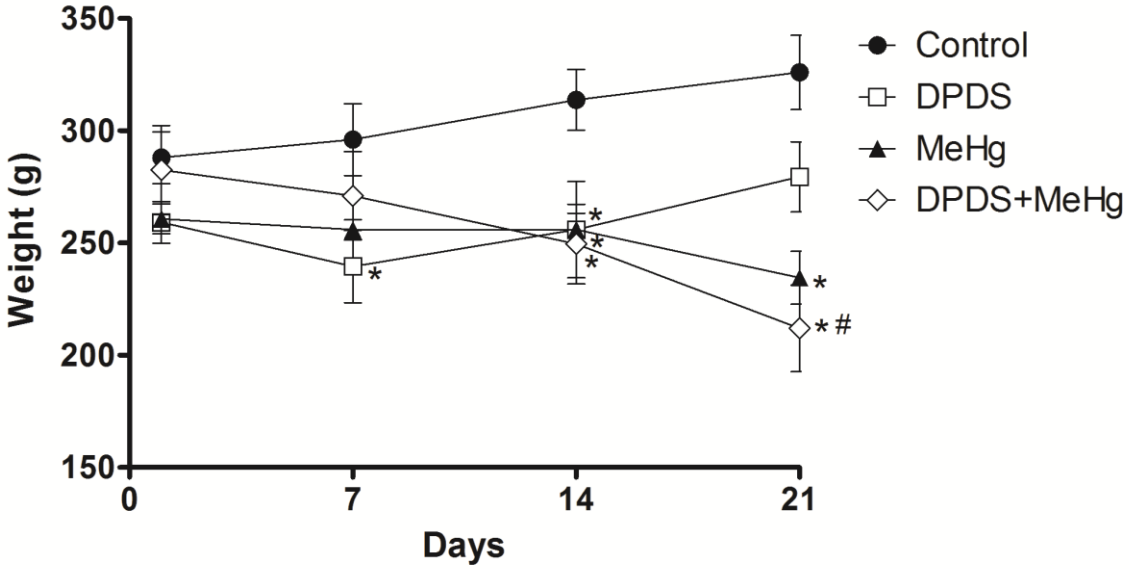

350
300
250
200
150
Weight (g)
0
7
14
21
Days
Control
DPDS
MeHg
DPDS+MeHg
*
*
*
*
*
*#

**Figure 2.**

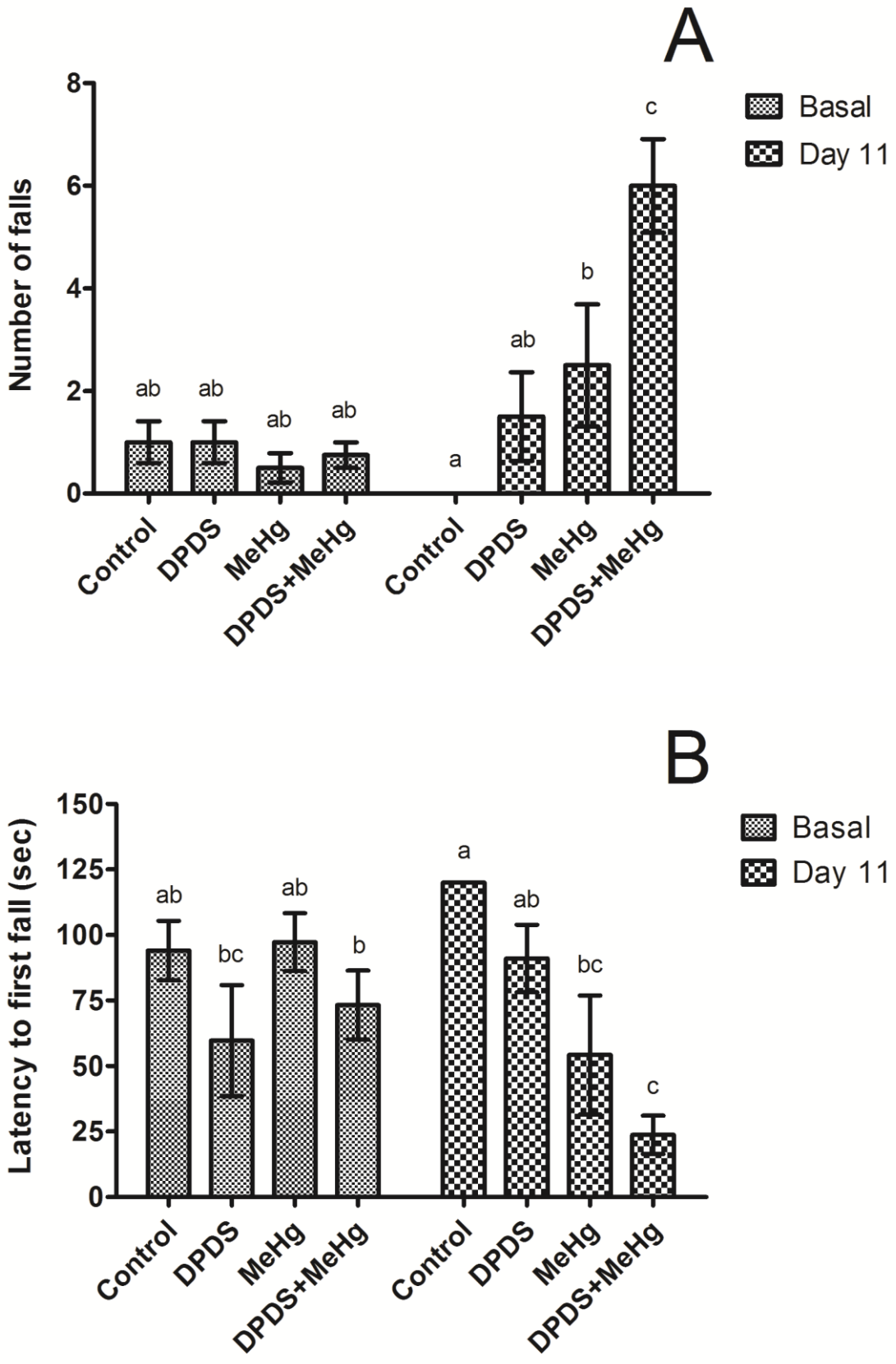

A
Number of falls
0
2
4
6
8
ab
ab
ab
ab
a
ab
b
c
Control
DPDS
MeHg
DPDS+MeHg
Control
DPDS
MeHg
DPDS+MeHg
Basal
Day 11
B
Latency to first fall (sec)
0
25
50
75
100
125
150
ab
bc
ab
b
a
ab
bc
c
Control
DPDS
MeHg
DPDS+MeHg
Control
DPDS
MeHg
DPDS+MeHg
Basal
Day 11

**Figure 3.**

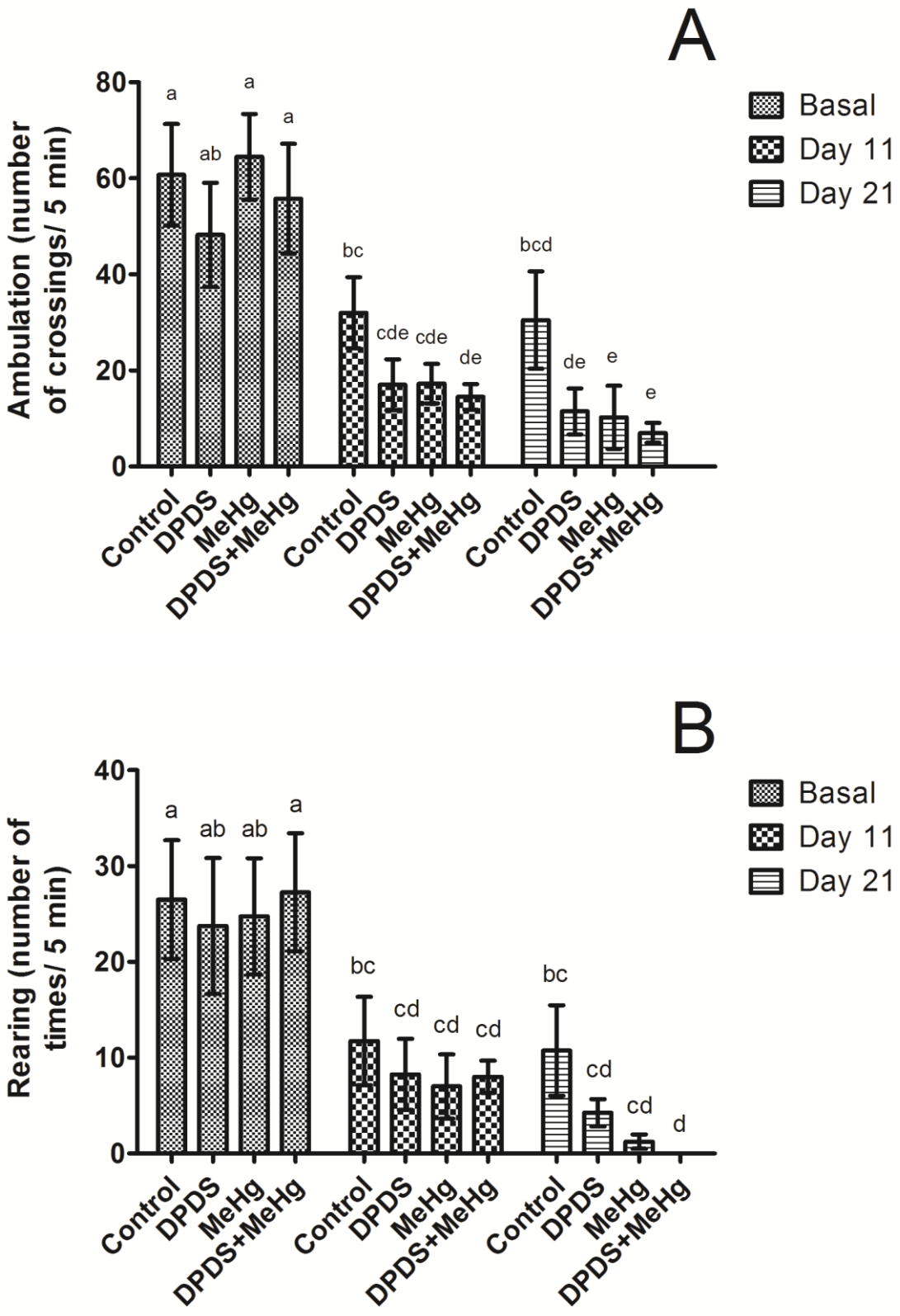
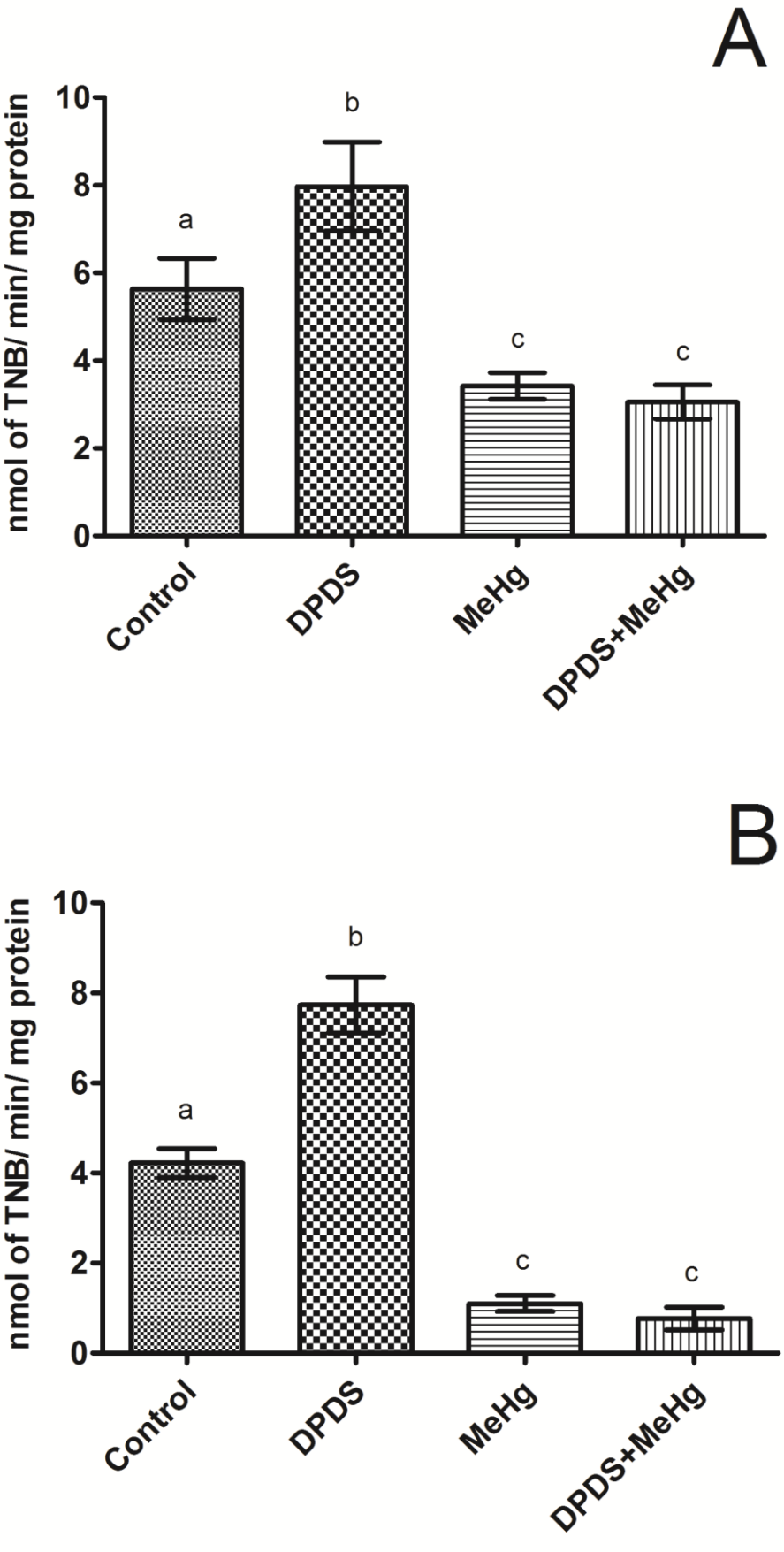

A
Ambulation (number of crossings/ 5 min)
80
60
40
20
0
a
ab
a
a
bc
cde
cde
de
bcd
de
e
e
Control
DPDS
MeHg
DPDS+MeHg
Basal
Day 11
Day 21
B
Rearing (number of times/ 5 min)
40
30
20
10
0
a
ab
ab
a
bc
cd
cd
cd
bc
cd
cd
d
Control
DPDS
MeHg
DPDS+MeHg
Basal
Day 11
Day 21

**Figure 4.**


A
nmol of TNB/ min/ mg protein
10
8
6
4
2
0
a
b
c
c
Control
DPDS
MeHg
DPDS+MeHg
B
nmol of TNB/ min/ mg protein
10
8
6
4
2
0
a
b
c
c
Control
DPDS
MeHg
DPDS+MeHg

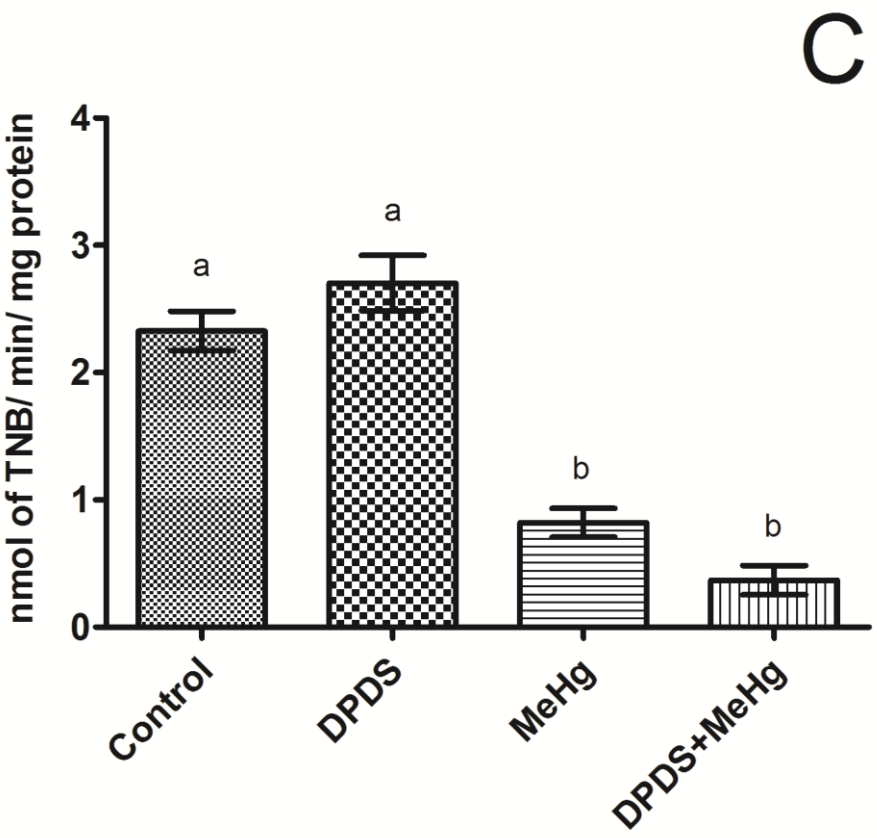

C
nmol of TNB/ min/ mg protein
4
3
2
1
0
a
a
b
b
Control
DPDS
MeHg
DPDS+MeHg

**Figure 5.**

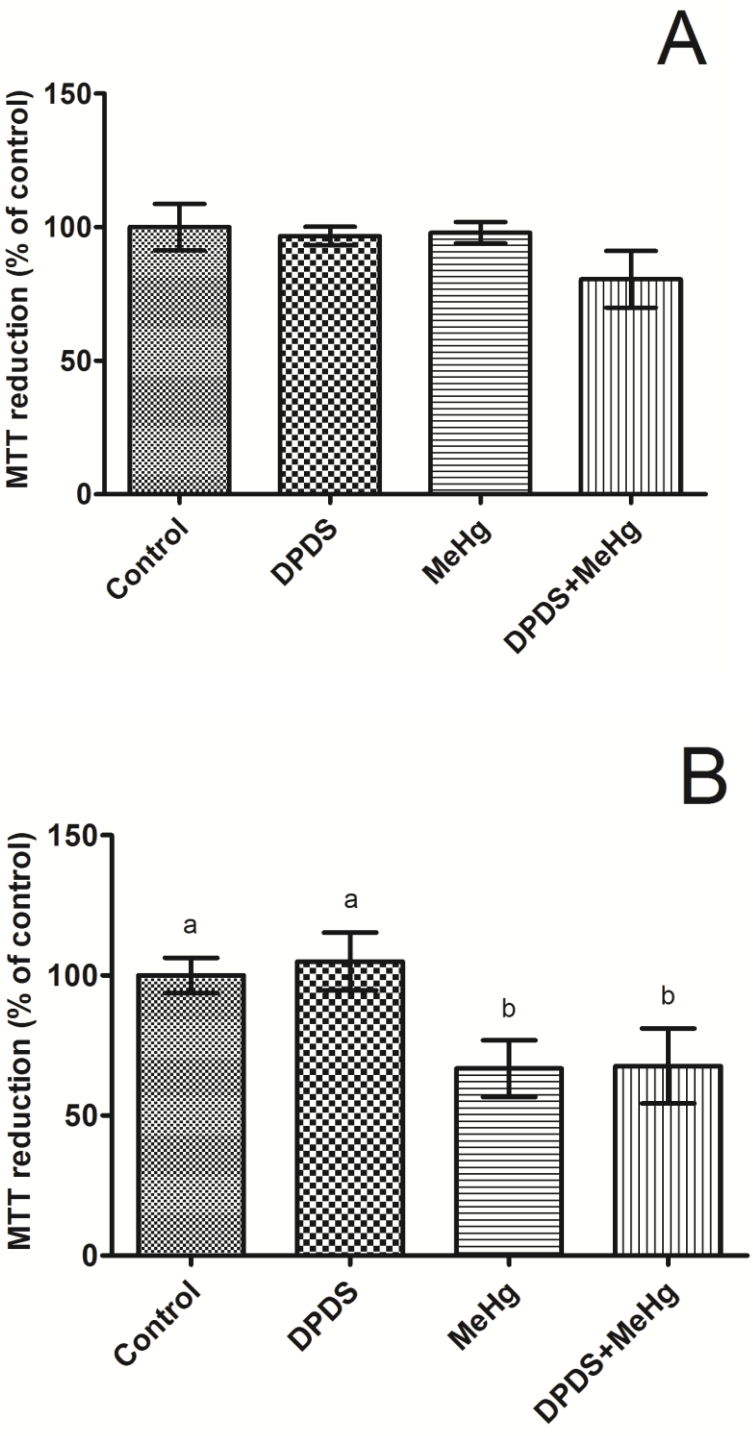

A
MTT reduction (% of control)
150
100
50
0
Control
DPDS
MeHg
DPDS+MeHg
B
MTT reduction (% of control)
150
100
50
0
a
a
b
b
Control
DPDS
MeHg
DPDS+MeHg

**Figure 6.**

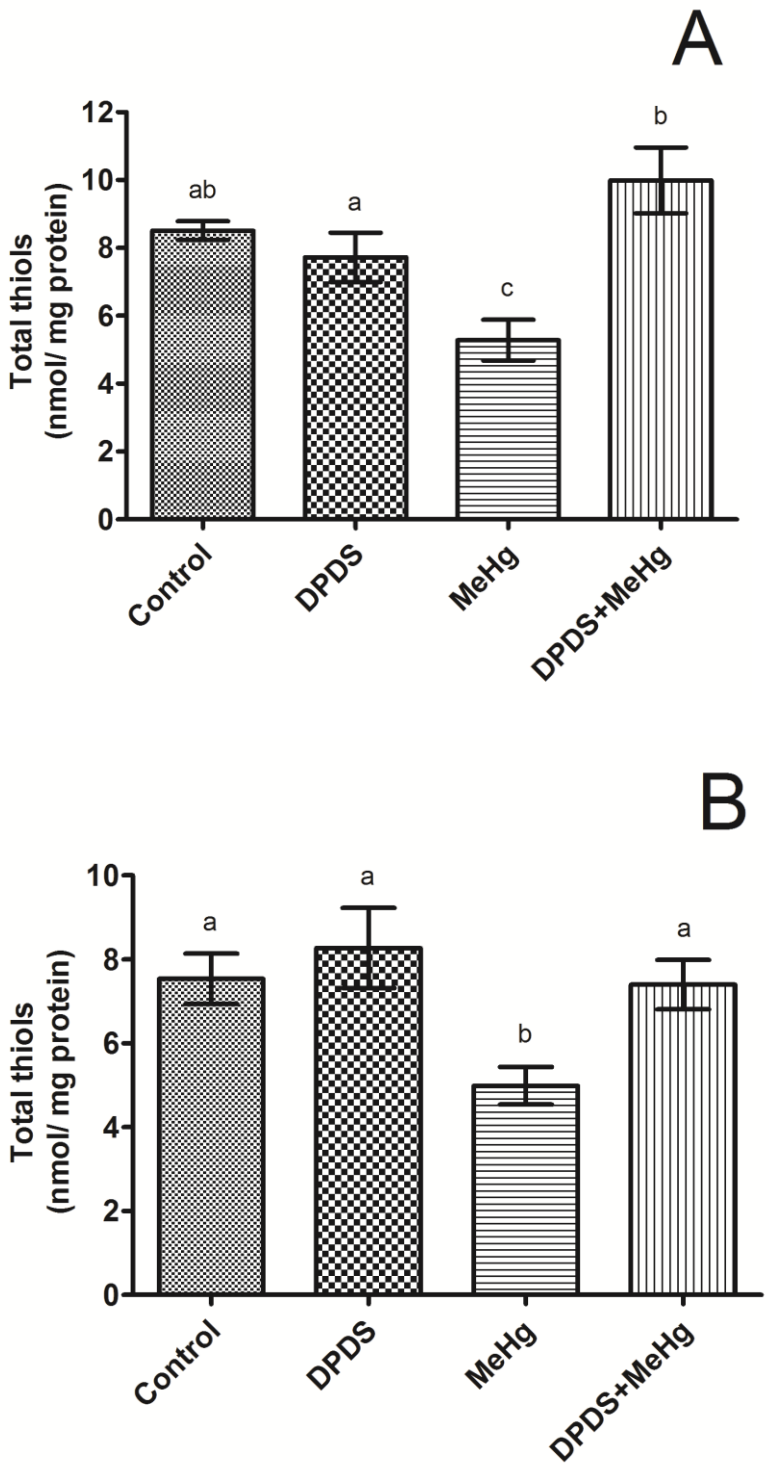

A
Total thiols (nmol/ mg protein)
12
10
8
6
4
2
0
ab
a
c
b
Control
DPDS
MeHg
DPDS+MeHg
B
Total thiols (nmol/ mg protein)
10
8
6
4
2
0
a
a
b
a
Control
DPDS
MeHg
DPDS+MeHg

**Figure 7.**

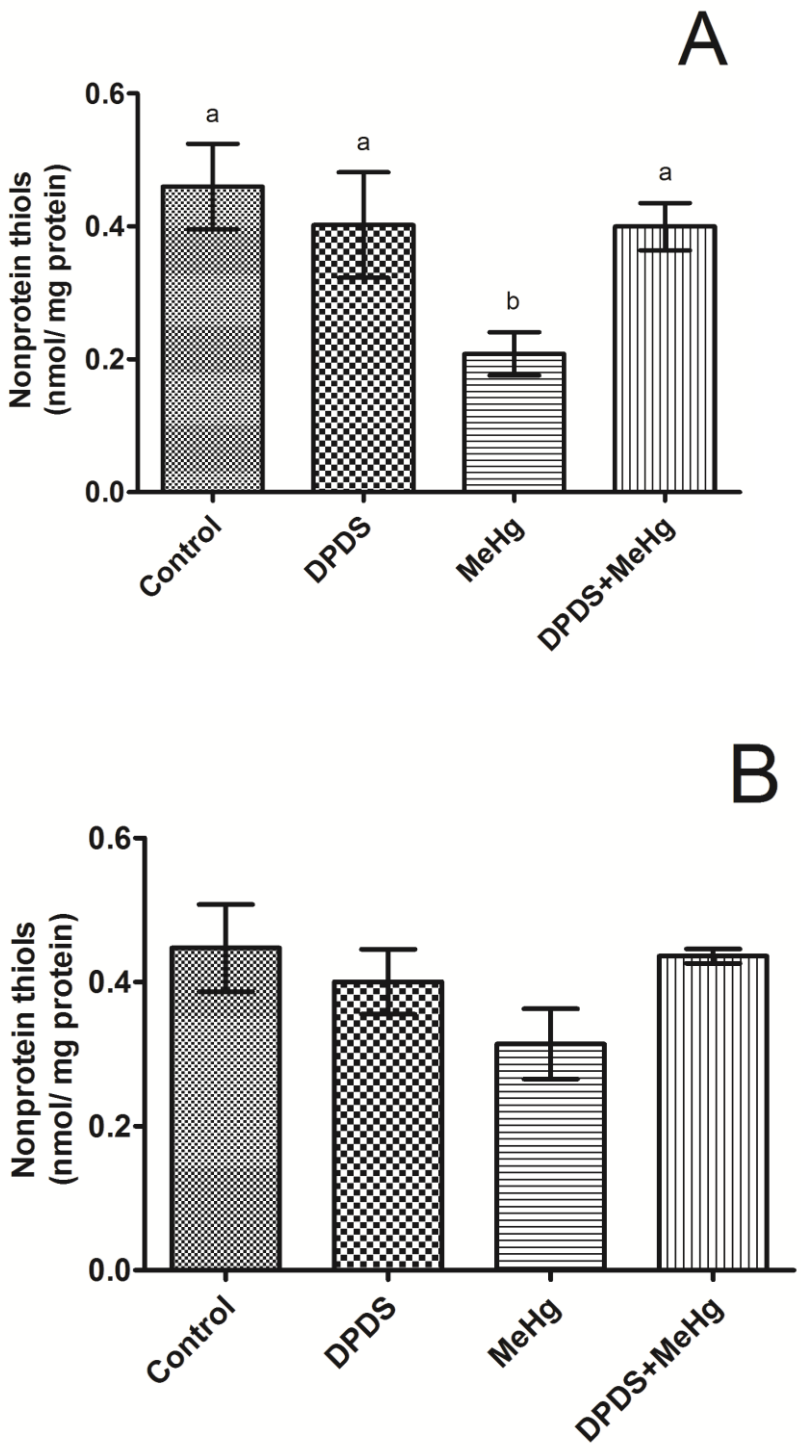

A
Nonprotein thiols (nmol/ mg protein)
0.6
0.4
0.2
0.0
a
a
b
a
Control
DPDS
MeHg
DPDS+MeHg
B
Nonprotein thiols (nmol/ mg protein)
0.6
0.4
0.2
0.0
Control
DPDS
MeHg
DPDS+MeHg

**Figure 8.**

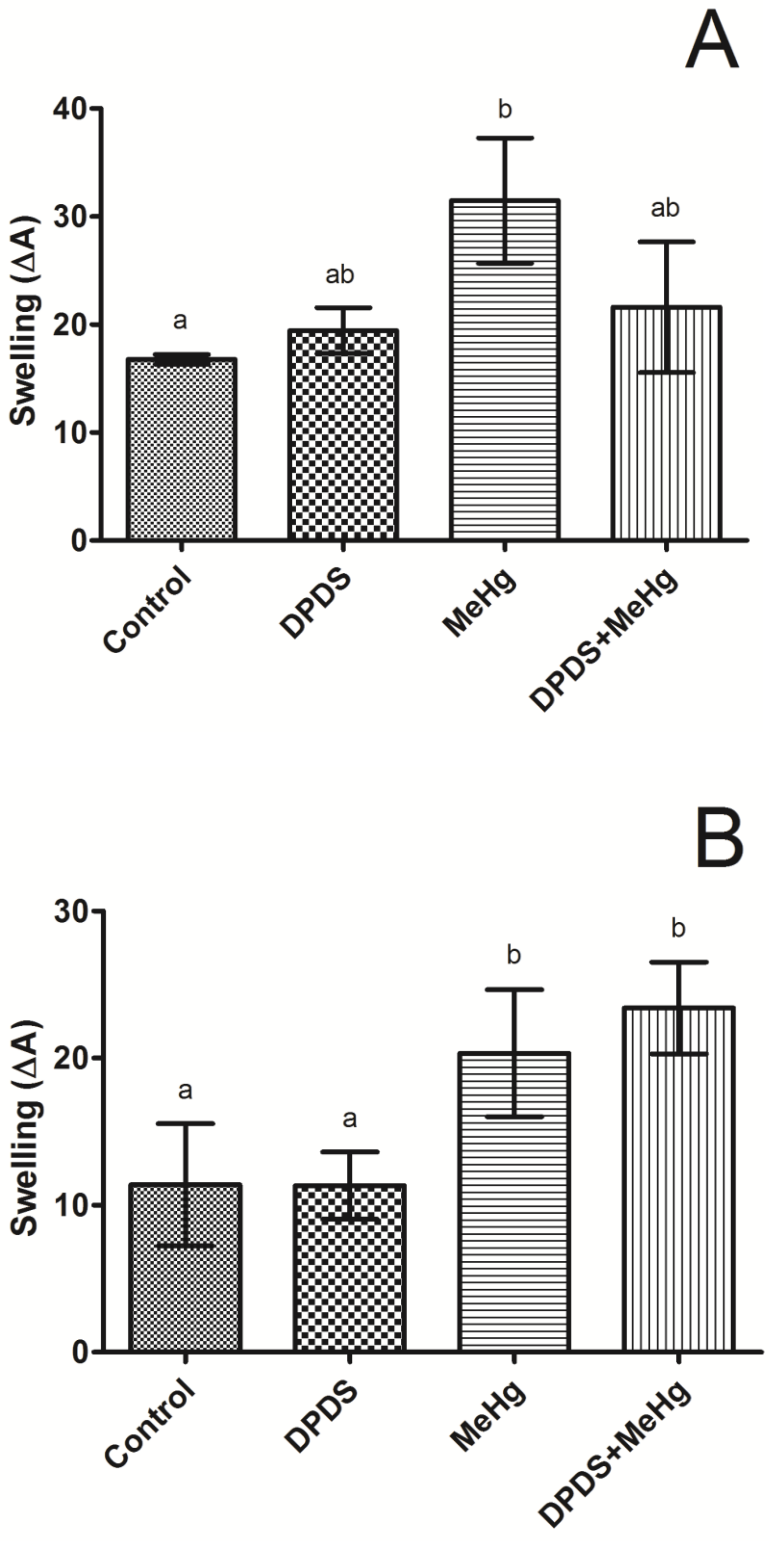

A
Swelling (ΔA)
40
30
20
10
0
a
ab
b
ab
Control
DPDS
MeHg
DPDS+MeHg
B
Swelling (ΔA)
30
20
10
0
a
a
b
b
Control
DPDS
MeHg
DPDS+MeHg

**Figure 9.**

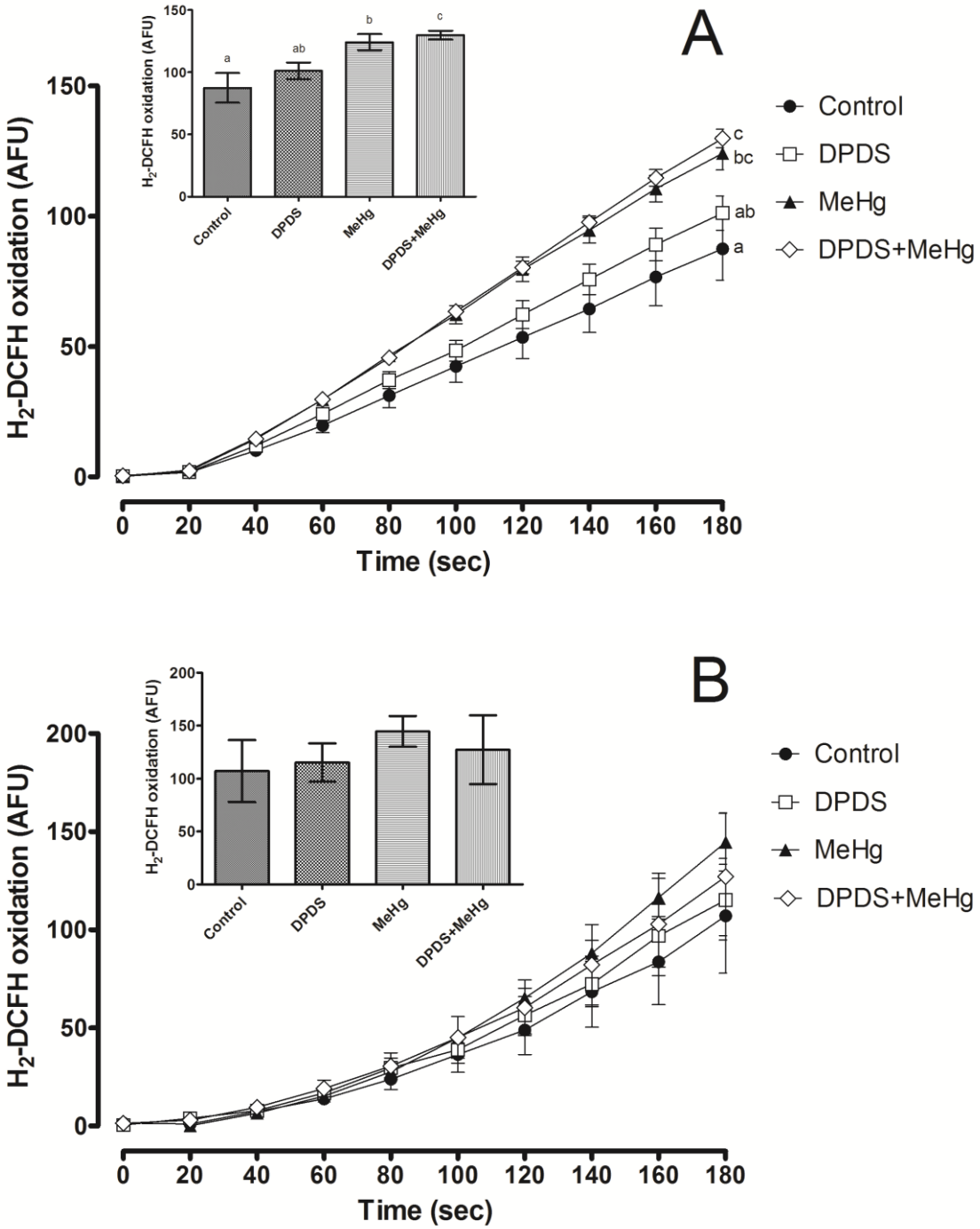

A
H2-DCFH oxidation (AFU)
Time (sec)
Control
DPDS
MeHg
DPDS+MeHg
a
ab
b
c
bc
B
H2-DCFH oxidation (AFU)
Time (sec)
Control
DPDS
MeHg
DPDS+MeHg

**Figure 10.**

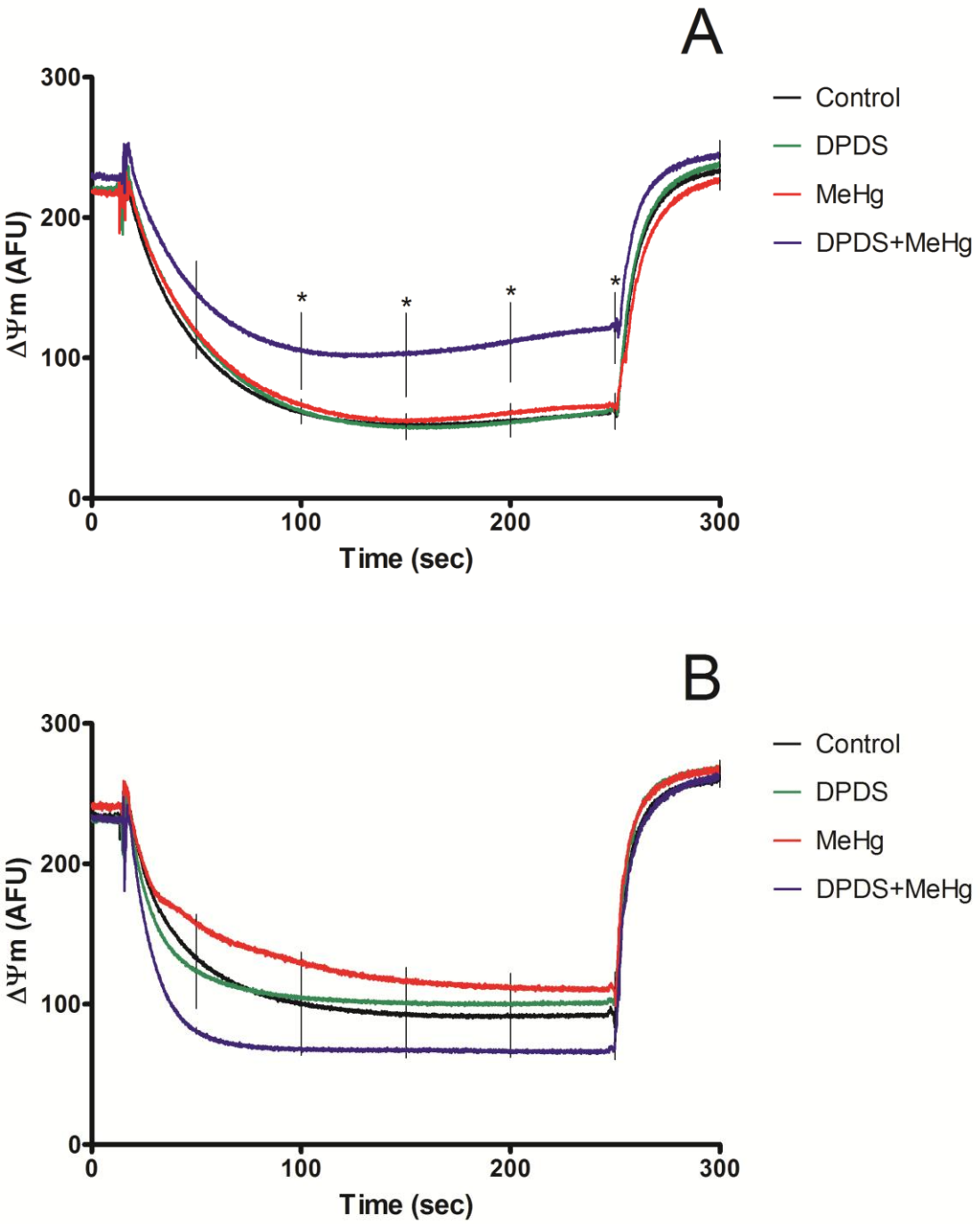
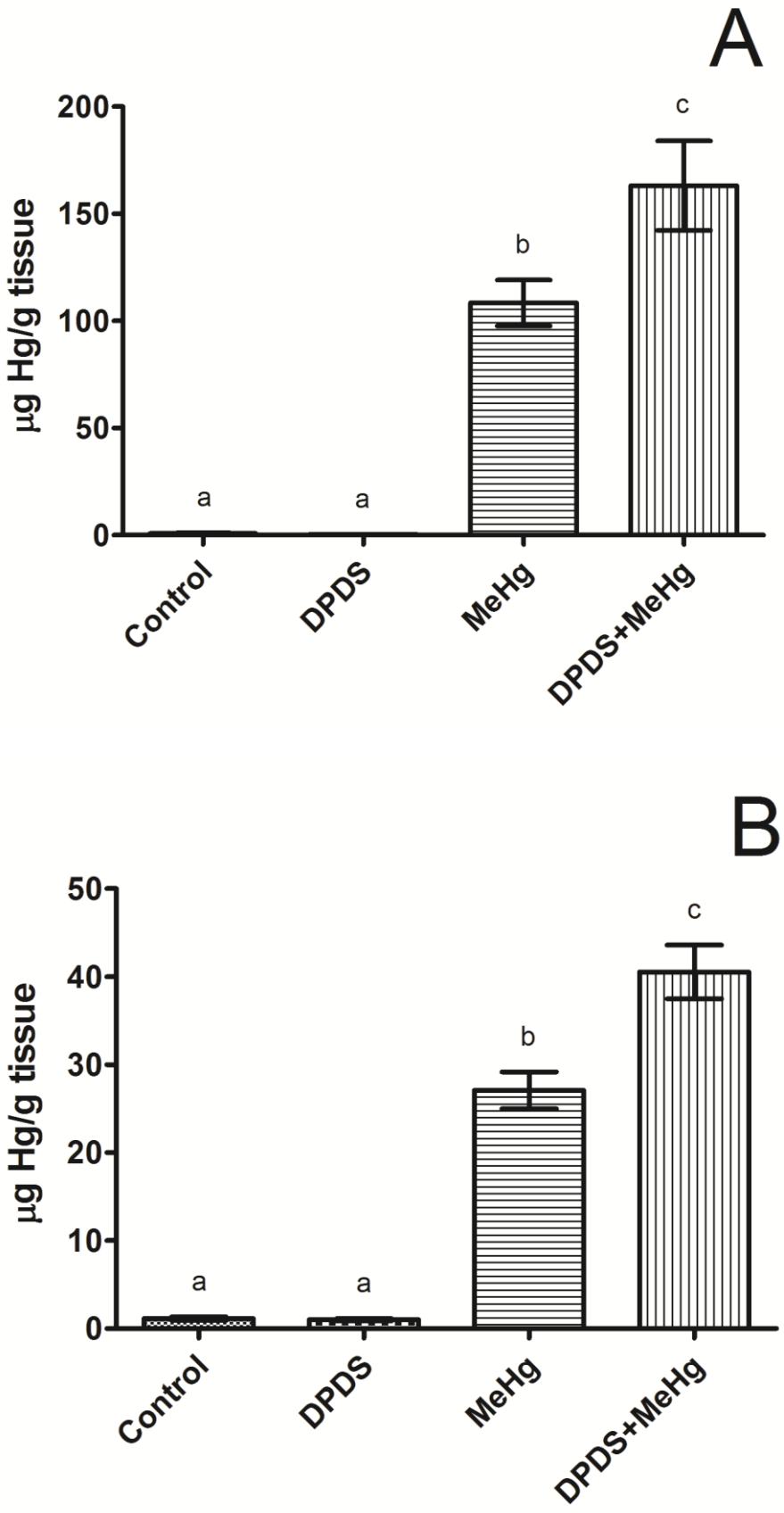
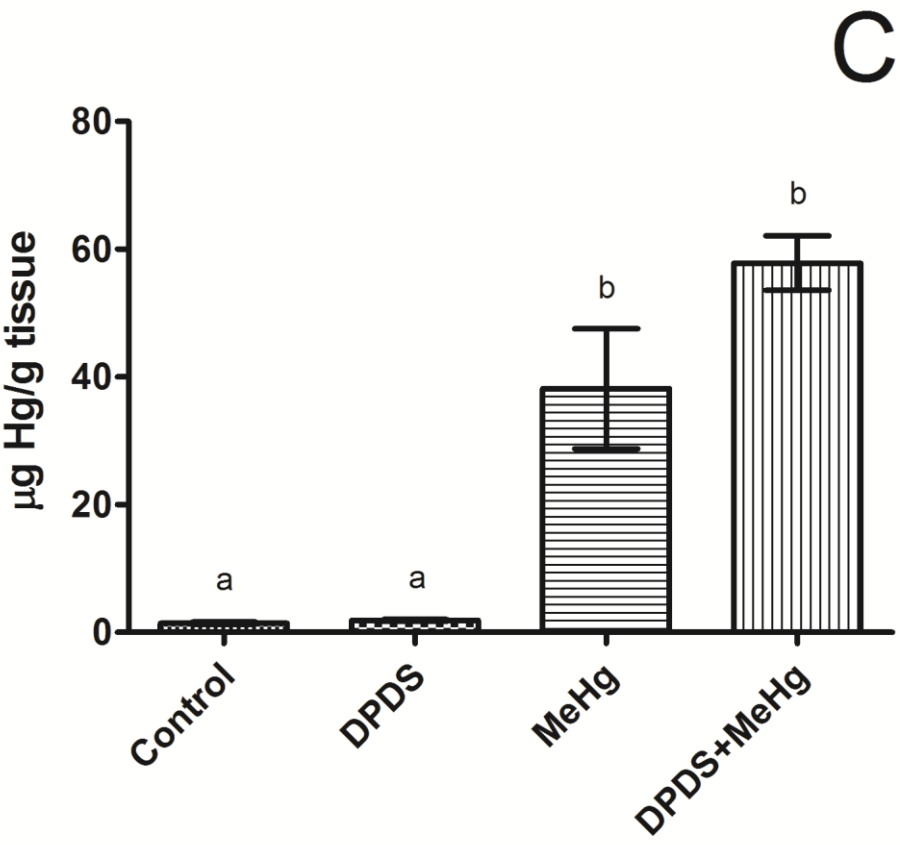

A
300
200
100
0
ΔΨm (AFU)
0
100
200
300
Time (sec)
*
*
*
*
Control
DPDS
MeHg
DPDS+MeHg
B
300
200
100
0
ΔΨm (AFU)
0
100
200
300
Time (sec)
Control
DPDS
MeHg
DPDS+MeHg

**Figure 11.**


A
200
150
100
50
0
µg Hg/g tissue
a
a
b
c
Control
DPDS
MeHg
DPDS+MeHg
B
50
40
30
20
10
0
µg Hg/g tissue
a
a
b
c
Control
DPDS
MeHg
DPDS+MeHg


C
80
60
40
20
0
µg Hg/g tissue
a
a
b
b
Control
DPDS
MeHg
DPDS+MeHg

## 3. DISCUSSÃO

Os compostos orgânicos de selênio têm sido propostos como potenciais agentes terapêuticos contra a formação e ações deletérias de EROs secundárias à uma variedade de patologias. Em particular o disseleneto de difenila e o ebselen, os quais que têm sido demonstrados como potentes agentes antioxidantes em uma variedade de modelos experimentais (Nogueira e Rocha, 2010; 2011). A excitotoxicidade glutamatérgica caracteriza-se pela superestimulação dos receptores glutamatérgicos o que por sua vez desencadeia uma sequência de eventos celulares que incluem o estresse oxidativo (Trotti e cols., 1998). Além disso, diversos estudos têm mostrado uma relação direta entre a produção de EROs, deficiências na captação de glutamato e doenças neurodegenerativas agudas e crônicas (Margaill e cols., 2005; Halliwell, 2006). Dessa forma, os antioxidantes são uma classe terapêutica promissora para o tratamento de doenças neurodegenerativas. No artigo 1 apresentado nesta tese avaliamos os efeitos do ebselen e do disseleneto de difenila, bem como, a associação destes compostos com um modulador do sistema glutamatérgico, a guanosina, na proteção do estresse oxidativo induzido por glutamato em fatias de córtex, estriado e hipocampo de rato. De fato, foi observado neste trabalho que o glutamato aumentou a produção de EROs bem como diminuiu a captação de [$^3$H]-glutamato em fatias estriatais, corticais e hipocampais de ratos. Frente a isso, observamos que tanto o ebselen quanto o disseleneto de difenila foram capazes de reduzir a produção de EROs induzida por glutamato em fatias de córtex, estriado e hipocampo de ratos. Estes resultados demonstram o potencial farmacológico de ambos os compostos em prevenir os primeiros sinais da neurotoxicidade glutamatérgica, tal como o estresse oxidativo.

Da mesma forma, a guanosina foi capaz de prevenir o aumento na geração de EROs induzida por glutamato em fatias estriatais, corticais e hipocampais. Estes resultados demonstram novamente a relação entre o aumento do glutamato extracelular e o aumento da produção de EROs, considerando que a guanosina também protegeu contra a inibição da captação de glutamato em todas as regiões do cérebro testadas. Estes resultados estão de acordo com outros estudos que vêm demonstrando que os efeitos neuroprotetores da guanosina poderiam estar relacionados com a sua habilidade de reduzir o glutamato extracelular pela ativação da captação de glutamato pelos astrócitos (Frizzo e cols., 2002; Soares e cols., 2004), com a subsequente prevenção da produção de EROs (Rathbone e cols., 1999; Ciccarelli e cols., 2001; Frizzo e cols., 2001; Frizzo e cols., 2002; Frizzo e cols., 2003; Schmidt e cols., 2005).

Também foi observado no artigo 1 que a combinação de guanosina com ebselen ou disseleneto de difenila foi mais efetiva em prevenir a produção de EROs induzida por glutamato em fatias estriatais, corticais e hipocampais do que o uso de cada composto separadamente. Isto provavelmente se deve ao fato de que esta combinação pode proporcionar proteção através de diferentes mecanismos: a guanosina atuaria aumentando a captação astrocitária de glutamato enquanto que os antioxidantes, ebeselen e disseleneto de difenila, por sua vez agiriam neutralizando as EROs geradas ou impediriam as reações de propagação dos radicais formados. Além disso, a combinação permite o uso de concentrações mais baixas de cada composto e, portanto, menos tóxicas. Sendo assim, os resultados do artigo 1 mostram que os compostos orgânicos de selênio em combinação com a guanosina são terapias promissoras contra o dano oxidativo induzido por glutamato em doenças do SNC.

Um dos mecanismos propostos para explicar a toxicidade do MeHg é o estresse oxidativo o qual poderia estar relacionado com alterações nas funções mitocondriais. Os resultados do manuscrito 1 mostraram que o MeHg causou disfunção mitocondrial em fatias de fígado de rato, a qual foi observada por uma diminuição na atividade de enzimas desidrogenases, um aumento na produção de EROs, uma diminuição no consumo de oxigênio e o colapso do $\Delta\Psi m$ mitocondrial. Estas descobertas estão de acordo com outros estudos corroborando a disfunção mitocondrial após a exposição ao MeHg tanto *in vivo* quanto *in vitro* (Mori e cols., 2007; Cambier e cols., 2009; Glaser e cols., 2010b; Wagner e cols., 2010b; Roos e cols., 2011; Yin e cols., 2011). Neste estudo também foi possível observar que o $Ca^{2+}$ aumentou a produção de EROS induzida pelo MeHg. O MeHg é conhecido por alterar a homeostase do $Ca^{2+}$ podendo causar a disfunção mitocondrial e levar ao aumento da produção de EROs (Aschner e cols., 2007). Baseado nestas observações, os resultados apresentados aqui são consistentes com uma diminuição no $\Delta\Psi m$ mitocondrial secundária a entrada de $Ca^{2+}$ induzida por MeHg durante a pré-incubação da fatia e com a subsequente superprodução de EROs.

Neste estudo o disseleneto de difenila foi capaz de prevenir a toxicidade mitocondrial induzida por MeHg em fatias de fígado a qual pode estar associada com a sua habilidade de restaurar a atividade de enzimas desidrogenases mitocondriais possivelmente por formar um complexo com MeHg assim reduzindo a carga total de MeHg na célula. Os resultados apresentados qui são consistentes com trabalhos prévios *in vivo* nos quais a exposição ao MeHg diminui a atividade de enzimas desidrogenases (Yoshino e cols., 1966; Glaser e cols., 2010b; Mori e cols., 2011). O consumo de oxigênio mitocondrial também foi diminuido após a exposição de fatias de fígado ao MeHg estando de acordo com a inibição de desidrogenases

pelo MeHg. O disseleneto de difenila preveniu parcialmente (nas concentrações de 1 e 5 µM) ou completamente (na concentração de 0.5 µM) a inibição do consumo de oxigênio mitocondrial induzida por MeHg, o que poderia estar associado com a modulação das desidrogenases da cadeia respiratória mitocondrial. O disseleneto de difenila também preveniu a perda do ΔΨm mitocondrial induzida por MeHg nas concentrações mais baixas, enquanto na sua concentração mais alta ele protegeu apenas parcialmente da despolarização mitocondrial induzida por MeHg. De fato, 5 µM de disseleneto de difenila causou a despolarização mitocondrial, o que provavelmente está relacionado a oxidação excessiva de grupos tióis críticos em proteínas mitocondriais (Puntel e cols., 2010). Da mesma forma, o disseleneto de difenila preveniu completamente a produção de EROs apenas na concentração mais baixa, causando um aumento na oxidação da DCFH em concentrações mais altas do que 1 µM indicando que estas poderiam oxidar grupos tióis importantes em proteinas mitocondriais e romper a integridade mitocondrial. Dessa forma, o DPDS pode diminuir a toxicidade do MeHg na mitocôndria através da sua redução a selenol/selenolato (PhSeH/PhSe−), o qual poderia bloquear diretamente os efeitos pró-oxidativos do MeHg devido a sua atividade tiol-peroxidase ou poderia ainda formar um complexo estável e inerte com o MeHg. No entanto, em altas concentrações os efeitos tóxicos do disseleneto de difenila deveriam ser considerados, uma vez que podem causar a oxidação de grupos –SH levando a inativação de proteínas tiólicas importantes.

Tendo em vista a proteção desempenhada pelo disseleneto de difenilla frente a disfunção mitocondrial induzida por MeHg (manuscrito 1), no manuscrito 2 investigou-se um possível efeito protetor do disseleneto de difenila sobre a toxicidade induzida por MeHg em ratos. Os resultados apresentados aqui mostram que a exposição ao MeHg *in vivo* induziu a disfunção mitocondrial a qual foi observada pelo aumento no inchaço mitocondrial, diminuição na atividade de desidrogenases mitocondriais no cérebro e, aumento no inchaço mitocondrial e produção de EROs no fígado. A afinidade do MeHg por grupos -SH é considerada um importante mecanismo na mediação da toxicidade do MeHg. Como comentado anteriormente, a ligação do MeHg aos grupos –SH pode inativar enzimas incluindo complexos da cadeia respiratória (Valko e cols., 2005; Glaser e cols., 2010a; Glaser e cols., 2010b; Farina e cols., 2011a; Farina e cols., 2011c; Mori e cols., 2011;), o que é consistente com a diminuição na atividade das desidrogenases mitocondriais de cérebro de ratos expostos ao MeHg observado aqui. Da mesma forma, a inibição destes complexos pode contribuir para o inchaço mitocondrial e para a produção de EROs na exposição ao MeHg. Por outro lado, o co-tratamento com disseleneto de difenila preveniu a depleção de grupos

tióis induzida por MeHg em mitocôndrias de cérebro e no fígado de ratos. Portanto, a prevenção da depleção de tióis pelo disseleneto de difenila parece novamente residir na sua habilidade para formar um complexo com o MeHg assim, efetivamente reduzindo a ligação de MeHg com proteínas e tióis livres. No entanto, ao contrário do estudo *in vitro* (manuscrito 1) o co-tratamento com disseleneto de difenila não protegeu da difunção mitocondrial induzida por MeHg em cérebro e fígado de ratos, sugerindo que outros mecanismos, além da interação com tióis, estão envolvidos na disfunção mitocondrial induzida por MeHg observada aqui.

O efeito do disseleneto de difenila frente à perda da coordenação motora em ratos induzida por MeHg também foi investigado no manuscrito 2. Os déficits motores são geralmente os efeitos neurológicos mais evidentes após a exposição ao MeHg (Kim e cols., 2000; Goulet e cols., 2003; Dietrich e cols., 2005; Rice, 1996). No entanto, como observado neste trabalho o co-tratamento com disseleneto de difenila aumentou a perda da coordenação motora (teste do rotarod) induzida por MeHg em ratos. Estes resultados indicam que o disseleneto de difenila aumentou os efeitos neurotóxicos induzidos pelo MeHg em ratos.

Os resultados apresentados aqui também mostraram que o disseleneto de difenila aumentou a deposição de Hg no cérebro e no fígado de ratos expostos ao MeHg. Uma possível explicação para esse aumento poderia ser a conversão de DPDS em ácido selenídrico, este composto de selênio pode se ligar ao MeHg para formar um complexo menos solúvel (Pinheiro et al., 2009). Dessa forma, o aumento na deposição de Hg hepática e cerebral pelo co-tratamento com disseleneto de difenila possivelmente envolve interações Hg:Se com a formação de um composto menos excretável que pode acumular em órgão críticos (Pinheiro et al., 2009). Estes resultados estão de acordo com outros estudos que mostram uma deposição elevada de Hg em regiões do cérebro após a administração oral de selênio (Prohaska e Ganther, 1977; Ganther, 1978; Moller-Madsen e Danscher, 1991; Schionning e cols., 1997; Schionning, 2000; Newland e cols., 2006).

Por fim, foi observado neste estudo que o tratamento com MeHg inibiu a atividade da TrxR de cérebro, fígado e rins de ratos o que está de acordo com outros estudos (Wagner e cols., 2010a; Branco e cols., 2011; Carvalho e cols., 2011). Uma vez que a TrxR é crítica para as defesas antioxidants celulares a inibiçao desta enzima poderia ter um papel central na toxicidade do MeHg. Recentemente, o disseleneto de difenila foi demonstrado ser substrato para a TrxR cerebral e hepática, o que poderia explicar pelo menos em parte as propriedades antioxidantes do disseleneto de difenila (de Freitas e cols., 2010; de Freitas e Rocha, 2011). Neste estudo, os ratos tratados apenas com disseleneto de difenila mostraram uma maior

atividade da TrxR renal e hepática. A formação de ácido selenídrico a partir do DPDS também poderia explicar o aumento da atividade da TrxR, uma vez que esta forma inorgânica de selênio pode ser convertida em selenocisteína e incorporada em selenoenzimas tais como a TrxR (Pinheiro et al., 2009). O co-tratamento com disseleneto de difenila e MeHg não foi capaz de proteger ou recuperar a atividade da TrxR inibida por MeHg em cérebro, fígado e rins de rato. De forma semelhante, estudos *in vitro* e *in vivo* mostraram que o selenito foi capaz de recuperar a atividade da TrxR quando inibida por $HgCl_2$ mas não por MeHg (Branco e cols., 2011; Carvalho e cols., 2011).

Em conjunto, os resultados apresentados aqui reforçam o papel central da disfunção mitocondrial na toxicidade induzida pelo MeHg tanto *in vitro* quanto *in vivo* e o papel da TrxR como um alvo molecular para o MeHg em ratos. Além disso, os resultados indicam que os compostos orgânicos de selênio, como o ebselen e o disseleneto de difenila, são agentes promissores contra o dano oxidativo induzido tanto por glutamato quanto por MeHg *in vitro*, no entanto deve-se ter muita cautela ao extrapolar estes resultados para situações *in vivo*, uma vez que o co-tratamento com disseleneto de difenila aumentou a neurotoxicidade do MeHg em ratos.

## 4. CONCLUSÕES

Artigo 1

1. Os compostos antioxidantes ebselen e disseleneto de difenila preveniram a produção de EROs induzida por glutamato em fatias de córtex, estriado e hipocampo de rato.

2. O modulador do sistema glutamatérgico guanosina, preveniu a geração de EROs induzida por glutamato em fatias de córtex, estriado e hipocampo de rato.

3. A guanosina protegeu frente a inibição da captação de [$^3$H]-glutamato em fatias de córtex, estriado e hipocampo de rato.

4. A combinação de um modulador do sistema glutamatérgico, a guanosina, e de antioxidantes (ebselen e disseleneto de difenila) foi mais efetiva em prevenir os efeitos pró-oxidantes do glutamato do que os compostos isolados.

Manuscrito 1

1. O disseleneto de difenila, em baixas concentrações, preveniu o aumento da produção de EROs induzidas por MeHg em mitocôndrias isoladas de fatias de fígado de rato.

2. O disseleneto de difenila preveniu a inibição da atividade de enzimas desidrogenases mitocondriais (redução do MTT) induzida por MeHg em fatias de fígado de rato.

3. O disseleneto de difenila, em baixas concentrações, protegeu frente a perda do $\Delta\psi m$ induzida por MeHg em mitocôndrias isoladas de fatias de fígado de rato.

4. O disseleneto de difenila, na concentração mais baixa, preveniu a redução do consumo de oxigênio induzido por MeHg em mitocôndrias isoladas de fatias de fígado de rato.

Manuscrito 2

1. O tratamento com disseleneto de difenila aumentou o déficit motor induzido por MeHg em ratos.

2. O disseleneto de difenila preveniu a depleção de tióis mitocondriais, mas não a disfunção mitocondrial induzida por MeHg em cérebro e fígado de rato *ex vivo*.

3. A exposição ao MeHg inibiu a atividade da TrxR em cérebro, fígado e rins de rato *ex vivo*. O tratamento com disseleneto de difenila aumentou a atividade da TrxR em fígado e rins, no entanto, o co-tratamento com disseleneto de difenila não preveniu a inibição da atividade da TrxR por MeHg em cérebro, fígado e rins de rato *ex vivo*.

4. O tratamento com disseleneto de difenila aumentou a deposição de Hg em cérebro e fígado de rato *ex vivo*.

## 5. PERSPECTIVAS

Com base nos resultados obtidos no presente trabalho, faz-se necessário:

- Investigar a combinação de ebselen ou disseleneto de difenila com a guanosina em reduzir os efeitos excitotóxicos do glutamato em modelos *in vivo*.

- Investigar os mecanismos envolvidos na proteção do disseleneto de difenila frente à disfunção mitocondrial induzida por MeHg.

- Estudar a interação entre MeHg e disseleneto de difenila para melhor entender os efeitos deste complexo em sistemas biológicos.

- Investigar se o disseleneto de difenila pode exercer efeitos protetores frente à disfunção mitocondrial em outros modelos animais.

## 6. REFERÊNCIAS BIBLIOGRÁFICAS


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, 2009.

AIZENMAN, E., LIPTON, S.A., LORING, R.H. Selective modulation of NMDA responses by reduction and oxidation. **Neuron**. 2**,** 1257-1263, 1989.

AIZENMAN, E., HARTNETT, K.A., REYNOLDS, I.J. Oxygen free radicals regulate NMDA receptor function via a redox modulatory site. **Neuron**. 5**,** 841-846, 1990.

ALBRECHT, J., TALBOT, M., KIMELBERG, H.K., ASCHNER, M. The role of sulfhydryl groups and calcium in the mercuric chloride-induced inhibition of glutamate uptake in rat primary astrocyte cultures. **Brain Res**. 607**,** 249-254, 1993.

ALI, S.F., LEBEL, C.P., BONDY, S.C. Reactive oxygen species formation as a biomarker of methylmercury and trimethyltin neurotoxicity. **Neurotoxicology**. 13**,** 637-648, 1992.

ALLAN, C.B., LACOURCIERE, G.M., STADTMAN, T.C. Responsiveness of selenoproteins to dietary selenium. **Annu Rev Nutr**. 19**,** 1-16, 1999.

ALLEN, J.W., SHANKER, G., TAN, K.H., ASCHNER, M. The consequences of methylmercury exposure on interactive functions between astrocytes and neurons. **Neurotoxicology**. 23**,** 755-759, 2002.

ANDERSON, C.M., SWANSON, R.A. Astrocyte glutamate transport: review of properties, regulation, and physiological functions. **Glia**. 32**,** 1-14, 2000.

ARNER, E.S.J., HOLMGREN, A. Physiological functions of thioredoxin and thioredoxin reductase. **Eur J Biochem.** 267**,** 6102-6109, 2000.

ARRIZA, J.L., ELIASOF, S., KAVANAUGH, M.P., AMARA, S.G. Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. **Proc Natl Acad Sci U S A**. 94**,** 4155-4160, 1997.

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

ARUNDINE, M., TYMIANSKI, M. Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. **Cell Calcium**. 34**,** 325-337, 2003.

ASCHNER, M., EBERLE, N.B., MILLER, K., KIMELBERG, H.K. Interactions of methylmercury with rat primary astrocyte cultures: inhibition of rubidium and glutamate uptake and induction of swelling. **Brain Res**. 530**,** 245-250, 1990.

ASCHNER, M., SYVERSEN, T., SOUZA, D.O., ROCHA, J.B., FARINA, M. Involvement of glutamate and reactive oxygen species in methylmercury neurotoxicity. **Braz J Med Biol Res**. 40**,** 285-291, 2007.

ASCHNER, M., ONISHCHENKO, N., CECCATELLI, S. Toxicology of alkylmercury compounds. **Met Ions Life Sci**. 7**,** 403-434, 2010.

ATCHISON, W.D., HARE, M.F. Mechanisms of methylmercury-induced neurotoxicity. **FASEB J**. 8**,** 622-629, 1994.

ATTWELL, D., BARBOUR, B., SZATKOWSKI, M. Nonvesicular release of neurotransmitter. **Neuron**. 11**,** 401-407, 1993.

ATTWELL, D., LAUGHLIN, S.B. An energy budget for signaling in the grey matter of the brain. **J Cereb Blood Flow Metab**. 21**,** 1133-1145, 2001.

BAKIR, F., DAMLUJI, S.F., AMIN-ZAKI, L., MURTADHA, M., KHALIDI, A., AL-RAWI, N.Y., TIKRITI, S., DAHAHIR, H.I., CLARKSON, T.W., SMITH, J.C., DOHERTY, R.A. Methylmercury poisoning in Iraq. **Science**. 181**,** 230-241, 1973.

BALLATORI, N., CLARKSON, T.W. Biliary secretion of glutathione and of glutathione-metal complexes. **Fundam Appl Toxicol**. 5**,** 816-831, 1985.

BARBOSA, N.B., ROCHA, J.B., ZENI, G., EMANUELLI, T., BEQUE, M.C., BRAGA, A.L. Effect of organic forms of selenium on delta-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., PEROTTONI, 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. D. NOGUEIRA, C. W. GUECHEVA, T. N. BELLINASO, M. D. ROCHA, J. B. T. Diphenyl diselenide supplementation delays the development of N-nitroso-N-methylurea-induced mammary tumors. **Arch Toxicol.** 82, 655-663, 2008.

BARON, B.M., DUDLEY, M.W., MCCARTY, D.R., MILLER, F.P., REYNOLDS, I.J., SCHMIDT, C.J. Guanine nucleotides are competitive inhibitors of N-methyl-D-aspartate at its receptor site both in vitro and in vivo. **J Pharmacol Exp Ther**. 250**,** 162-169, 1989.

BILLUPS, B., ROSSI, D., ATTWELL, D. Anion conductance behavior of the glutamate uptake carrier in salamander retinal glial cells. **J Neurosci**. 16**,** 6722-6731, 1996.

BITTIGAU, P., IKONOMIDOU, C. Glutamate in neurologic diseases. **J Child Neurol**. 12**,** 471-485, 1997.

BONVENTO, G., SIBSON, N., PELLERIN, L. Does glutamate image your thoughts? **Trends Neurosci**. 25**,** 359-364, 2002.

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., NOGUEIRA, C. W., PANATIERI, R. B., ROCHA, J. B. T., ZENI, G., Acute liver damage induced by 2-nitropropane in rats: effect of diphenyl diselenide on antioxidant defenses. **Chem Biol Interact.** 160, 99-107, 2006.

BRADFORD, H.F. Glutamate, GABA and epilepsy. **Prog Neurobiol**. 47**,** 477-511, 1995.

BRAGADIN, M., MARTON, D., MANENTE, S., GRASSO, M., TONINELLO, A. Methylmercury induces the opening of the permeability transition pore in rat liver mitochondria. **J Inorg Biochem**. 89**,** 159-162, 2002.

BRANCO, V., CANARIO, J., HOLMGREN, A., CARVALHO, C. Inhibition of the thioredoxin system in the brain and liver of zebra-seabreams exposed to waterborne methylmercury. **Toxicol Appl Pharmacol**. 251**,** 95-103, 2011.

BRANDÃO, R., SANTOS, F.W., ZENI, G., ROCHA, J.B., NOGUEIRA, C.W. DMPS and N-acetylcysteine induced renal toxicity in mice exposed to mercury. **Biometals**. 19**,** 389-398, 2006.

BRANDÃO, R., BORGES, L.P., DE OLIVEIRA, R., ROCHA, J.B., NOGUEIRA, C.W. Diphenyl diselenide protects against hematological and immunological alterations induced by mercury in mice. **J Biochem Mol Toxicol**. 22**,** 311-319, 2008.

BRANDÃO, R., ACKER, C. I., LEITE, M. R., BARBOSA, N. B. V., NOGUEIRA, C. W. Diphenyl diselenide protects against glycerol-induced renal damage in rats. **J Appl Toxicol.** 29, 612-618, 2009.

BRANDÃO, R., MORESCO, R.N., BELLÉ, L.P., LEITE, M.R., DE FREITAS, M.L., BIANCHINI, A., NOGUEIRA, C.W. Diphenyl diselenide potentiates nephrotoxicity induced by mercuric chloride in mice. **J Appl Toxicol.** 31, 773-782, 2010.

BROOKES, N. Specificity and reversibility of the inhibition by HgCl2 of glutamate transport in astrocyte cultures. **J Neurochem**. 50**,** 1117-1122, 1988.

BRORSON, J.R., ZHANG, Z., VANDENBERGHE, W. Ca(2+) permeation of AMPA receptors in cerebellar neurons expressing glu receptor 2. **J Neurosci**. 19**,** 9149-9159, 1999.

BRUNO, V., BATTAGLIA, G., COPANI, A., D'ONOFRIO, M., DI IORIO, P., DE BLASI, A., MELCHIORRI, D., FLOR, P.J., NICOLETTI, F. Metabotropic glutamate receptor

subtypes as targets for neuroprotective drugs. **J Cereb Blood Flow Metab**. 21**,** 1013-1033, 2001.

BURGER, M. E., FACHINETTO, R., WAGNER, C., PEROTTONI, J., PEREIRA, R. P., ZENI, G. ROCHA, J. B. T. Effects of diphenyl-diselenide on orofacial dyskinesia model in rats. **Brain Res Bull.** 70, 165-170, 2006.

BURGOS, J.S., BARAT, A., SOUZA, D.O., RAMIREZ, G. Guanine nucleotides protect against kainate toxicity in an ex vivo chick retinal preparation. **FEBS Lett**. 430**,** 176-180, 1998.

BURGOS, J.S., BARAT, A., RAMIREZ, G. Guanine nucleotides block agonist-driven 45Ca2+ influx in chick embryo retinal explants. **Neuroreport**. 11**,** 2303-2305, 2000.

CAMBIER, S., BENARD, G., MESMER-DUDONS, N., GONZALEZ, P., ROSSIGNOL, R., BRETHES, D., BOURDINEAUD, J.P. At environmental doses, dietary methylmercury inhibits mitochondrial energy metabolism in skeletal muscles of the zebra fish (Danio rerio). **Int J Biochem Cell Biol**. 41**,** 791-799, 2009.

CARVALHO, C.M., MATOS, A.I., MATEUS, M.L., SANTOS, A.P., BATOREU, M.C. High-fish consumption and risk prevention: assessment of exposure to methylmercury in Portugal. **J Toxicol Environ Health A**. 71**,** 1279-1288, 2008.

CARVALHO, C.M., LU, J., ZHANG, X., ARNER, E.S., HOLMGREN, A. Effects of selenite and chelating agents on mammalian thioredoxin reductase inhibited by mercury: implications for treatment of mercury poisoning. **FASEB J**. 25**,** 370-381, 2011.

CHAUDHRY, F.A., LEHRE, K.P., VAN LOOKEREN CAMPAGNE, M., OTTERSEN, O.P., DANBOLT, N.C., STORM-MATHISEN, J. Glutamate transporters in glial plasma membranes: highly differentiated localizations revealed by quantitative ultrastructural immunocytochemistry. **Neuron**. 15**,** 711-720, 1995.

CHOI, D.W. Ionic dependence of glutamate neurotoxicity. **J Neurosci**. 7**,** 369-379, 1987.

CHOI, D.W. Excitotoxic cell death. **J Neurobiol**. 23**,** 1261-1276, 1992.

CHOI, D.W. Calcium and excitotoxic neuronal injury. **Ann N Y Acad Sci**. 747**,** 162-171, 1994.

CICCARELLI, R., BALLERINI, P., SABATINO, G., RATHBONE, M.P., D'ONOFRIO, M., CACIAGLI, F., DI IORIO, P. Involvement of astrocytes in purine-mediated reparative processes in the brain. **Int J Dev Neurosci**. 19**,** 395-414, 2001.

CLARKSON, T.W. The pharmacology of mercury compounds. **Annu Rev Pharmacol**. 12**,** 375-406, 1972.

CLARKSON, T.W., MAGOS, L., MYERS, G.J. The toxicology of mercury--current exposures and clinical manifestations. **N Engl J Med**. 349**,** 1731-1737, 2003.

COLLINGRIDGE, G.L., LESTER, R.A. Excitatory amino acid receptors in the vertebrate central nervous system. **Pharmacol Rev**. 41**,** 143-210, 1989.

COYLE, J.T., BIRD, S.J., EVANS, R.H., GULLEY, R.L., NADLER, J.V., NICKLAS, W.J., OLNEY, J.W. Excitatory amino acid neurotoxins: selectivity, specificity, and mechanisms of action. Based on an NRP one-day conference held June 30, 1980. **Neurosci Res Program Bull**. 19**,** 1-427, 1981.

DANBOLT, N.C. Glutamate uptake. **Prog Neurobiol**. 65**,** 1-105, 2001.

DAVIS, L.E., KORNFELD, M., MOONEY, H.S., FIEDLER, K.J., HAALAND, K.Y., ORRISON, W.W., CERNICHIARI, E., CLARKSON, T.W. Methylmercury poisoning: long-term clinical, radiological, toxicological, and pathological studies of an affected family. **Ann Neurol**. 35**,** 680-688, 1994.

DAWSON, D.A., MASAYASU, H., GRAHAM, D.I., MACRAE, I.M. The neuroprotective efficacy of ebselen (a glutathione peroxidase mimic) on brain damage induced by transient focal cerebral ischaemia in the rat. **Neurosci Lett**. 185**,** 65-69, 1995.

DE BEM, A. F., PORTELLA, R. D., COLPO, E., DUARTE, M. M. M. F., FREDIANE, A., TAUBE, P. S., NOGUEIRA, C. W., FARINA, M., DA SILVA, E. L., ROCHA, J. B. T. Diphenyl diselenide decreases serum levels of total cholesterol and tissue oxidative stress in cholesterol-fed rabbits. **Basic Clin Pharmacol Toxicol.** 105, 17-23, 2009.

DE FREITAS, A.S., FUNCK, V.R., ROTTA MDOS, S., BOHRER, D., MORSCHBACHER, V., PUNTEL, R.L., NOGUEIRA, C.W., FARINA, M., ASCHNER, M., ROCHA, J.B. Diphenyl diselenide, a simple organoselenium compound, decreases methylmercury-induced cerebral, hepatic and renal oxidative stress and mercury deposition in adult mice. **Brain Res Bull**. 79**,** 77-84, 2009.

DE FREITAS, A.S., PRESTES, A.D., WAGNER, C., SUDATI, J.H., ALVES, D., PORCIUNCULA, L.O., KADE, I.J., ROCHA, J.B.T. Reduction of Diphenyl Diselenide and Analogs by Mammalian Thioredoxin Reductase Is Independent of Their Gluthathione Peroxidase-Like Activity: A Possible Novel Pathway for Their Antioxidant Activity. **Molecules**. 15**,** 7699-7714, 2010.

DE FREITAS, A.S., ROCHA, J.B. Diphenyl diselenide and analogs are substrates of cerebral rat thioredoxin reductase: a pathway for their neuroprotective effects. **Neurosci Lett**. 503**,** 1-5, 2011.

DEUTCH, A.Y., ROTH, R.H. Neurotransmitters. Em: Fundamental Neuroscience, eds. Zigmond, M.J., Bloom, F.E., Landis, S.C., Roberts, J.L., Squire, L.R. Academic Press, San Diego, 1999.

DIAZ, D., KREJSA, C.M., WHITE, C.C., KEENER, C.L., FARIN, F.M., KAVANAGH, T.J. Tissue specific changes in the expression of glutamate-cysteine ligase mRNAs in mice exposed to methylmercury. **Toxicol Lett**. 122**,** 119-129, 2001.

DIETRICH, M.O., MANTESE, C.E., ANJOS, G.D., SOUZA, D.O., FARINA, M. Motor impairment induced by oral exposure to methylmercury in adult mice. **Environ Toxicol Pharmacol**. 19**,** 169-175, 2005.

DUTCZAK, W.J., BALLATORI, N. gamma-Glutamyltransferase-dependent biliary-hepatic recycling of methyl mercury in the guinea pig. **J Pharmacol Exp Ther**. 262**,** 619-623, 1992.

DUTCZAK, W.J., BALLATORI, N. Transport of the glutathione-methylmercury complex across liver canalicular membranes on reduced glutathione carriers. **J Biol Chem**. 269**,** 9746-9751, 1994.

DUVOISIN, R.M., ZHANG, C., RAMONELL, K. A novel metabotropic glutamate receptor expressed in the retina and olfactory bulb. **J Neurosci**. 15**,** 3075-3083, 1995.

FAIRMAN, W.A., VANDENBERG, R.J., ARRIZA, J.L., KAVANAUGH, M.P., AMARA, S.G. An excitatory amino-acid transporter with properties of a ligand-gated chloride channel. **Nature**. 375**,** 599-603, 1995.

FARINA, M., SOARES, F.A., ZENI, G., SOUZA, D.O., ROCHA, J.B. Additive pro-oxidative effects of methylmercury and ebselen in liver from suckling rat pups. **Toxicol Lett**. 146**,** 227-235, 2004.

FARINA, M., CAMPOS, F., VENDRELL, I., BERENGUER, J., BARZI, M., PONS, S., SUNOL, C. Probucol increases glutathione peroxidase-1 activity and displays long-lasting protection against methylmercury toxicity in cerebellar granule cells. **Toxicol Sci**. 112**,** 416-426, 2009.

FARINA, M., ASCHNER, M., ROCHA, J.B. Oxidative stress in MeHg-induced neurotoxicity. **Toxicol Appl Pharmacol**. 256**,** 405-417, 2011a.

FARINA, M., ROCHA, J.B., ASCHNER, M. Mechanisms of methylmercury-induced neurotoxicity: Evidence from experimental studies. **Life Sci**. 2011b.

FLOHE, L., GUNZLER, W.A., SCHOCK, H.H. Glutathione peroxidase: a selenoenzyme. **FEBS Lett**. 32**,** 132-134, 1973.

FLOHE, L. Glutathione peroxidase. **Basic Life Sci**. 49**,** 663-668, 1988.

FLORA, S.J., MITTAL, M., MEHTA, A. Heavy metal induced oxidative stress & its possible reversal by chelation therapy. **Indian J Med Res**. 128**,** 501-523, 2008.

FOLMER, V., BOLZAN, R.C., FARINA, M., ZENI, G., NOGUEIRA, C.W., EMANUELLI, T., ROCHA, J.B. Mechanism of delta-aminolevulinate dehydratase inhibition by phenyl selenoacetylene involves its conversion to diphenyl diselenide. **Toxicology**. 206**,** 403-411, 2005.

FONNUM, F. Glutamate: a neurotransmitter in mammalian brain. **J Neurochem**. 42**,** 1-11, 1984.

FRANCO, J.L., BRAGA, H.C., STRINGARI, J., MISSAU, F.C., POSSER, T., MENDES, B.G., LEAL, R.B., SANTOS, A.R., DAFRE, A.L., PIZZOLATTI, M.G., FARINA, M. Mercurial-induced hydrogen peroxide generation in mouse brain mitochondria: protective effects of quercetin. **Chem Res Toxicol**. 20**,** 1919-1926, 2007.

FRANCO, J.L., POSSER, T., DUNKLEY, P.R., DICKSON, P.W., MATTOS, J.J., MARTINS, R., BAINY, A.C.D., MARQUES, M.R., DAFRE, A.L., FARINA, M. Methylmercury neurotoxicity is associated with inhibition of the antioxidant enzyme glutathione peroxidase. **Free Radic Biol Med.** 47**,** 449-457, 2009.

FRANKE, K.W. A new toxicant occurring naturally in certain samples of plant foodstuVs: I. Results obtained in preliminary feeding trials. **J Nutr.** 8, 597–608, 1934a.

FRANKE, K.W. A new toxicant occurring naturally in certain samples of plant foodstuVs: II. The occurrence of the toxicant in the protein fraction. **J Nutr.** 8, 609–613, 1934b.

FRANKE, K.W., PAINTER, E.P. Selenium in proteins from toxic foodstuVs: IV. The eVect of feeding toxic proteins, toxic protein hydrolysates, and toxic protein hydrolysates from which the selenium has been removed. **J Nutr.** 10, 599–611, 1935.

FRIZZO, M.E., LARA, D.R., DAHM, K.C., PROKOPIUK, A.S., SWANSON, R.A., SOUZA, D.O. Activation of glutamate uptake by guanosine in primary astrocyte cultures. **Neuroreport**. 12**,** 879-881, 2001.

FRIZZO, M.E., LARA, D.R., PROKOPIUK ADE, S., VARGAS, C.R., SALBEGO, C.G., WAJNER, M., SOUZA, D.O. Guanosine enhances glutamate uptake in brain cortical slices at normal and excitotoxic conditions. **Cell Mol Neurobiol**. 22**,** 353-363, 2002.

FRIZZO, M.E., ANTUNES SOARES, F.A., DALL'ONDER, L.P., LARA, D.R., SWANSON, R.A., SOUZA, D.O. Extracellular conversion of guanine-based purines to guanosine specifically enhances astrocyte glutamate uptake. **Brain Res**. 972**,** 84-89, 2003.

GANTHER, H.E. Modification of methylmercury toxicity and metabolism by selenium and vitamin E: possible mechanisms. **Environ Health Perspect**. 25**,** 71-76, 1978.

GHISLENI, G., KAZLAUCKAS, V., BOTH, F.L., PAGNUSSAT, N., MIORANZZA, S., ROCHA, J.B.T., SOUZA, D.O., PORCIUNCULA, L.O. Diphenyl diselenide exerts anxiolytic-like effect in Wistar rats: putative roles of GABAA and 5HT receptors. **Prog Neuro-Psychopharmacol Biol Psychiatry** 32, 1508-1515, 2008.

GLASER, V., LEIPNITZ, G., STRALIOTTO, M.R., OLIVEIRA, J., DOS SANTOS, V.V., WANNMACHER, C.M.D., DE BERN, A.F., ROCHA, J.B.T., FARINA, M., LATINI, A. Oxidative stress-mediated inhibition of brain creatine kinase activity by methylmercury. **Neurotoxicology**. 31**,** 454-460, 2010a.

GLASER, V., NAZARI, E.M., MULLER, Y.M., FEKSA, L., WANNMACHER, C.M., ROCHA, J.B., DE BEM, A.F., FARINA, M., LATINI, A. Effects of inorganic selenium administration in methylmercury-induced neurotoxicity in mouse cerebral cortex. **Int J Dev Neurosci**. 28**,** 631-637, 2010b.

GOTZ, M.E., KUNIG, G., RIEDERER, P., YOUDIM, M.B. Oxidative stress: free radical production in neural degeneration. **Pharmacol Ther**. 63**,** 37-122, 1994.

GOULET, S., DORE, F.Y., MIRAULT, M.E. Neurobehavioral changes in mice chronically exposed to methylmercury during fetal and early postnatal development. **Neurotoxicol Teratol**. 25**,** 335-347, 2003.

GRAFF, R.D., FALCONER, M.M., BROWN, D.L., REUHL, K.R. Altered sensitivity of posttranslationally modified microtubules to methylmercury in differentiating embryonal carcinoma-derived neurons. **Toxicol Appl Pharmacol**. 144**,** 215-224, 1997.

GREENE, J.G., GREENAMYRE, J.T. Bioenergetics and glutamate excitotoxicity. **Prog Neurobiol**. 48**,** 613-634, 1996.

GUL, S., BAHADIR, B., HANCI, V., ACIKGOZ, S., BEKTAS, S., UGURBAS, E., ANKARALI, H., KALAYCI, M., ACIKGOZ, B. Effects of ebselen versus nimodipine on cerebral vasospasm subsequent to experimental subarachnoid hemorrhage in rats. **J Clin Neurosci**. 17**,** 608-611, 2010.

HALLIWELL, B. Oxidative stress and neurodegeneration: where are we now? **J Neurochem**. 97**,** 1634-1658, 2006.

HAMACHER, J., STAMMBERGER, U., WEBER, E., LUCAS, R., WENDEL, A. Ebselen improves ischemia-reperfusion injury after rat lung transplantation. **Lung**. 187**,** 98-103, 2009.

HARADA, M. Congenital Minamata disease: intrauterine methylmercury poisoning. **Teratology**. 18**,** 285-288, 1978.

HARADA, M. Neurotoxicity of methylmercury: Minamata and the Amazon. In: Yasui, M., Strong, M.J., Ota, K., Verity, M.A., eds. Mineral and metal neurotoxicology. London: CRC Press; p. 177–188, 1997.

HAUGETO, O., ULLENSVANG, K., LEVY, L.M., CHAUDHRY, F.A., HONORE, T., NIELSEN, M., LEHRE, K.P., DANBOLT, N.C. Brain glutamate transporter proteins form homomultimers. **J Biol Chem**. 271**,** 27715-27722, 1996.

HEADLEY, P.M., GRILLNER, S. Excitatory amino acids and synaptic transmission: the evidence for a physiological function. **Trends Pharmacol Sci**. 11**,** 205-211, 1990.

HEATH, P.R., SHAW, P.J. Update on the glutamatergic neurotransmitter system and the role of excitotoxicity in amyotrophic lateral sclerosis. **Muscle Nerve**. 26**,** 438-458, 2002.

HINTELMANN, H. Organomercurials. Their formation and pathways in the environment. **Met Ions Life Sci**. 7**,** 365-401, 2010.

HOLLMANN, M., HARTLEY, M., HEINEMANN, S. Ca2+ permeability of KA-AMPA--gated glutamate receptor channels depends on subunit composition. **Science**. 252**,** 851-853, 1991.

HUDSPITH, M.J. Glutamate: a role in normal brain function, anaesthesia, analgesia and CNS injury. **Br J Anaesth**. 78**,** 731-747, 1997.

HUETTNER, J.E. Kainate receptors and synaptic transmission. **Prog. Neurobiol.** 70, 387-407, 2003.

HUETTNER, J.E. Glutamate receptor channels in rat DRG neurons: activation by kainate and quisqualate and blockade of desensitization by Con A. **Neuron.** 5, 255-266, 1990.

HUNTER, D., RUSSELL, D.S. Focal cerebellar and cerebellar atrophy in a human subject due to organic mercury compounds. **J Neurol Neurosurg Psychiatry**. 17**,** 235-241, 1954.

IMAI, H., MASAYASU, H., DEWAR, D., GRAHAM, D.I., MACRAE, I.M. Ebselen protects both gray and white matter in a rodent model of focal cerebral ischemia. **Stroke**. 32**,** 2149-2154, 2001.

INEU, R.P., PEREIRA, M.E., ASCHNER, M., NOGUEIRA, C.W., ZENI, G., ROCHA, J.B.T. Diphenyl diselenide reverses gastric lesions in rats: Involvement of oxidative stress. **Food Chem Toxicol**. 46, 3023-3029, 2008.

INSUG, O., DATAR, S., KOCH, C.J., SHAPIRO, I.M., SHENKER, B.J. Mercuric compounds inhibit human monocyte function by inducing apoptosis: evidence for formation of reactive oxygen species, development of mitochondrial membrane permeability transition and loss of reductive reserve. **Toxicology**. 124**,** 211-224, 1997.

KANAI, Y., HEDIGER, M.A. Primary structure and functional characterization of a high-affinity glutamate transporter. **Nature**. 360**,** 467-471, 1992.

KANAI, Y., HEDIGER, M.A. The glutamate and neutral amino acid transporter family: physiological and pharmacological implications. **Eur J Pharmacol**. 479**,** 237-247, 2003.

KAUR, P., ASCHNER, M., SYVERSEN, T. Glutathione modulation influences methyl mercury induced neurotoxicity in primary cell cultures of neurons and astrocytes. **Neurotoxicology**. 27**,** 492-500, 2006.

KERPER, L.E., BALLATORI, N., CLARKSON, T.W. Methylmercury transport across the blood-brain barrier by an amino acid carrier. **Am J Physiol**. 262**,** R761-765, 1992.

KIM, C.Y., NAKAI, K., KASANUMA, Y., SATOH, H. Comparison of neurobehavioral changes in three inbred strains of mice prenatally exposed to methylmercury. **Neurotoxicol Teratol**. 22**,** 397-403, 2000.

KLECKNER, N.W., DINGLEDINE, R. Requirement for glycine in activation of NMDA-receptors expressed in Xenopus oocytes. **Science**. 241**,** 835-837, 1988.

KWAK, S., WEISS, J.H. Calcium-permeable AMPA channels in neurodegenerative disease and ischemia. **Curr Opin Neurobiol**. 16**,** 281-287, 2006.

LAPCHAK, P.A., ZIVIN, J.A. Ebselen, a seleno-organic antioxidant, is neuroprotective after embolic strokes in rabbits: synergism with low-dose tissue plasminogen activator. **Stroke**. 34**,** 2013-2018, 2003.

LARA, D.R., SCHMIDT, A.P., FRIZZO, M.E., BURGOS, J.S., RAMIREZ, G., SOUZA, D.O. Effect of orally administered guanosine on seizures and death induced by glutamatergic agents. **Brain Res**. 912**,** 176-180, 2001.

LEBEL, C.P., ALI, S.F., MCKEE, M., BONDY, S.C. Organometal-induced increases in oxygen reactive species: the potential of 2',7'-dichlorofluorescin diacetate as an index of neurotoxic damage. **Toxicol Appl Pharmacol.** 104, 17–24, 1990.

LEI, S.Z., PAN, Z.H., AGGARWAL, S.K., CHEN, H.S., HARTMAN, J., SUCHER, N.J., LIPTON, S.A. Effect of nitric oxide production on the redox modulatory site of the NMDA receptor-channel complex. **Neuron**. 8**,** 1087-1099, 1992.

LILLIG, C.H., HOLMGREN, A. Thioredoxin and related molecules - From biology to health and disease. **Antioxid Redox Signal**. 9**,** 25-47, 2007.

LIMKE, T.L., HEIDEMANN, S.R., ATCHISON, W.D. Disruption of intraneuronal divalent cation regulation by methylmercury: are specific targets involved in altered neuronal

development and cytotoxicity in methylmercury poisoning? **Neurotoxicology**. 25**,** 741-760, 2004.

LU, J., HOLMGREN, A. Selenoproteins. **J Biol Chem**. 284**,** 723-727, 2009.

LUCAS, D.R., NEWHOUSE, J.P. The toxic effect of sodium L-glutamate on the inner layers of the retina. **AMA Arch Ophthalmol**. 58**,** 193-201, 1957.

MACIEL, E.N., BOLZAN, R.C., BRAGA, A.L., ROCHA, J.B. Diphenyl diselenide and diphenyl ditelluride differentially affect delta-aminolevulinate dehydratase from liver, kidney, and brain of mice. **J Biochem Mol Toxicol**. 14**,** 310-319, 2000.

MAIORINO, M., ROVERI, A., COASSIN, M., URSINI, F. Kinetic mechanism and substrate specificity of glutathione peroxidase activity of ebselen (PZ51). **Biochem Pharmacol**. 37**,** 2267-2271, 1988.

MAIORINO, M., ROVERI, A., URSINI, F. Antioxidant effect of Ebselen (PZ 51): peroxidase mimetic activity on phospholipid and cholesterol hydroperoxides vs free radical scavenger activity. **Arch Biochem Biophys**. 295**,** 404-409, 1992.

MARAGAKIS, N.J., ROTHSTEIN, J.D. Glutamate transporters: animal models to neurologic disease. **Neurobiol Dis**. 15**,** 461-473, 2004.

MARGAILL, I., PLOTKINE, M., LEROUET, D. Antioxidant strategies in the treatment of stroke. **Free Radic Biol Med**. 39**,** 429-443, 2005.

MARTY, M.S., ATCHISON, W.D. Pathways mediating Ca2+ entry in rat cerebellar granule cells following in vitro exposure to methyl mercury. **Toxicol Appl Pharmacol**. 147**,** 319-330, 1997.

MARTY, M.S., ATCHISON, W.D. Elevations of intracellular Ca2+ as a probable contributor to decreased viability in cerebellar granule cells following acute exposure to methylmercury. **Toxicol Appl Pharmacol**. 150**,** 98-105, 1998.

MEINERZ, D.F., DE PAULA, M.T., COMPARSI, B., SILVA, M.U., SCHMITZ, A.E., BRAGA, H.C., TAUBE, P.S., BRAGA, A.L., ROCHA, J.B.T., DAFRE, A.L., FARINA, M., FRANCO J.L., POSSER, T. Protective effects of organoselenium compounds against methylmercury-induced oxidative stress in mouse brain mitochondrial-enriched fractions. **Braz J Med Biol Res.** 44, 1156-1163, 2011.

MELDRUM, B. Protection against ischaemic neuronal damage by drugs acting on excitatory neurotransmission. **Cerebrovasc Brain Metab Rev**. 2**,** 27-57, 1990.

MELDRUM, B., GARTHWAITE, J. Excitatory amino acid neurotoxicity and neurodegenerative disease. **Trends Pharmacol Sci**. 11**,** 379-387, 1990.

MELDRUM, B.S. Glutamate as a neurotransmitter in the brain: review of physiology and pathology. **J Nutr**. 130**,** 1007S-1015S, 2000.

MEOTTI, F.C., BORGES, V.C., ZENI, G., ROCHA, J.B., NOGUEIRA, C.W. Potential renal and hepatic toxicity of diphenyl diselenide, diphenyl ditelluride and Ebselen for rats and mice. **Toxicol Lett**. 143**,** 9-16, 2003.

MOLLER-MADSEN, B., DANSCHER, G. Localization of mercury in CNS of the rat. IV. The effect of selenium on orally administered organic and inorganic mercury. **Toxicol Appl Pharmacol**. 108**,** 457-473, 1991.

MORETTO, M.B., ROSSATO, J.I., NOGUEIRA, C.W., ZENI, G., ROCHA, J.B.T. Voltage-dependent ebselen and diorganochalcogenides inhibition of $^{45}Ca^{2+}$ influx into brain synaptosomes. **J. Biochem Mol Toxicol.** 17, 154-160, 2003.

MORETTO, M.B., FUNCHAL, C., SANTOS, A.Q., GOTTFRIED, C., BOFF, B., ZENI, G., PUREUR, R.P., SOUZA, D.O., WOFCHUK, S., ROCHA, J.B. Ebselen protects glutamate uptake inhibition caused by methyl mercury but does not by Hg2+. **Toxicology**. 214**,** 57-66, 2005.

MORETTO, M.B., THOMAZI, A.P., GODINHO, G., ROESSLER, T.M., NOGUEIRA, C. W., SOUZA, D.O., WOFCHUK, S., ROCHA, J.B.T. Ebselen and diorganylchalcogenides decrease in vitro glutamate uptake by rat brain slices: prevention by DTT and GSH. **Toxicol Vitro** 21, 639-645, 2007.

MORI, N., YASUTAKE, A., HIRAYAMA, K. Comparative study of activities in reactive oxygen species production/defense system in mitochondria of rat brain and liver, and their susceptibility to methylmercury toxicity. **Arch Toxicol**. 81**,** 769-776, 2007.

MORI, N., YASUTAKE, A., MARUMOTO, M., HIRAYAMA, K. Methylmercury inhibits electron transport chain activity and induces cytochrome c release in cerebellum mitochondria. **J Toxicol Sci**. 36**,** 253-259, 2011.

MORIN, D., ZINI, R., LIGERET, H., NECKAMEYER, W., LABIDALLE, S., TILLEMENT, J.P. Dual effect of ebselen on mitochondrial permeability transition. **Biochem Pharmacol**. 65**,** 1643-1651, 2003.

MOXON, A.L., RHIAN, M. Selenium poisoning. **Physiol Rev**. 23, 305–337, 1943.

MOTTET, N.K., SHAW, C.M., BURBACHER, T.M. Health risks from increases in methylmercury exposure. **Environ Health Perspect**. 63**,** 133-140, 1985.

MUGESH, G., PANDA, A., SINGH, H.B., PUNEKAR, N.S., BUTCHER, R.J. Glutathione peroxidase-like antioxidant activity of diaryl diselenides: a mechanistic study. **J Am Chem Soc**. 123**,** 839-850, 2001.

MULLE, C., SAILER, A., SWANSON, G.T., BRANA, C., O'GORMAN, S., BETTLER, B., HEINEMANN, S.F. Subunit composition of kainate receptors in hippocampal interneurons. **Neuron**. 28**,** 475-484, 2000.

MULLER, A., CADENAS, E., GRAF, P., SIES, H. A novel biologically active seleno-organic compound--I. Glutathione peroxidase-like activity in vitro and antioxidant capacity of PZ 51 (Ebselen). **Biochem Pharmacol**. 33**,** 3235-3239, 1984.

MUNDY, W.R., FREUDENRICH, T.M. Sensitivity of immature neurons in culture to metal-induced changes in reactive oxygen species and intracellular free calcium. **Neurotoxicology**. 21**,** 1135-1144, 2000.

NAKANISHI, S. Molecular diversity of glutamate receptors and implications for brain function. **Science**. 258**,** 597-603, 1992.

NAVARRO-ALARCON, M., CABRERA-VIQUE, C. Selenium in food and the human body: a review. **Sci Total Environ**. 400**,** 115-141, 2008.

NEWLAND, M.C., REED, M.N., LEBLANC, A., DONLIN, W.D. Brain and blood mercury and selenium after chronic and developmental exposure to methylmercury. **Neurotoxicology**. 27**,** 710-720, 2006.

NICHOLLS, D., ATTWELL, D. The release and uptake of excitatory amino acids. **Trends Pharmacol Sci**. 11**,** 462-468, 1990.

NOGUEIRA, C.W., ROTTA, L.N., PERRY, M.L., SOUZA, D.O., ROCHA, J.B.T. Diphenyl diselenide and diphenyl ditelluride affect the rat glutamatergic system in vitro and in vivo. **Brain Res.** 906, 157-163, 2001.

NOGUEIRA, C.W., ROTTA, L.N., ZENI, G., SOUZA, D.O., ROCHA, J.B.T. Exposure to ebselen changes glutamate uptake and release by rat brain synaptosomes. **Neurochem Res.** 27, 283-288, 2002.

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

NOGUEIRA, C.W., ZENI, G., ROCHA, J.B. Organoselenium and organotellurium compounds: toxicology and pharmacology. **Chem Rev**. 104**,** 6255-6285, 2004.

NOGUEIRA, C.W., ROCHA, J.B.T. Diphenyl diselenide: a Janus faced compound. **J. Braz. Chem. Soc.** 21, 2055–2071, 2010.

NOGUEIRA, C.W., ROCHA, J.B. Toxicology and pharmacology of selenium: emphasis on synthetic organoselenium compounds. **Arch Toxicol**. 85**,** 1313-1359, 2011.

NOWAK, L., BREGESTOVSKI, P., ASCHER, P., HERBET, A., PROCHIANTZ, A. Magnesium gates glutamate-activated channels in mouse central neurones. **Nature**. 307**,** 462-465, 1984.

OGAWA, A., YOSHIMOTO, T., KIKUCHI, H., SANO, K., SAITO, I., YAMAGUCHI, T., YASUHARA, H. Ebselen in acute middle cerebral artery occlusion: a placebo-controlled, double-blind clinical trial. **Cerebrovasc Dis**. 9**,** 112-118, 1999.

OGITA, K., YONEDA, Y. Characterization of $Na^+$-dependent binding sites of [3H]glutamate in synaptic membranes from rat brain. **Brain Res**. 397**,** 137-144, 1986.

OH, S.H., GANTHER, H.E., HOEKSTRA, W.G. Selenium as a component of glutathione periodase isolated from ovine erythrocytes. **Biochemistry**. 13**,** 1825-1829, 1974.

OKAMOTO, N., HORI, S., AKAZAWA, C., HAYASHI, Y., SHIGEMOTO, R., MIZUNO, N., NAKANISHI, S. Molecular characterization of a new metabotropic glutamate receptor mGluR7 coupled to inhibitory cyclic AMP signal transduction. **J Biol Chem**. 269**,** 1231-1236, 1994.

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

OLNEY, J.W. Brain lesions, obesity, and other disturbances in mice treated with monosodium glutamate. **Science**. 164**,** 719-721, 1969.

OYAMA, Y., YAMAZAKI, Y., OKADA, Y., TAKAHAMA, K., SATOH, M., HAYASHI, H. Toxicity of methylmercury conjugated with L-cysteine on rat thymocytes and human leukemia K562 cells in comparison with that of methylmercury chloride. **Environ Toxicol Pharmacol**. 9**,** 49-55, 2000.

OZAKI, M., NAKAMURA, M., TERAOKA, S., OTA, K. Ebselen, a novel anti-oxidant compound, protects the rat liver from ischemia-reperfusion injury. **Transpl Int**. 10**,** 96-102, 1997.

OZAWA, S., KAMIYA, H., TSUZUKI, K. Glutamate receptors in the mammalian central nervous system. **Prog Neurobiol**. 54**,** 581-618, 1998.

PALMER, C.L., COTTON, L., HENLEY, J.M. The molecular pharmacology and cell biology of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors. **Pharmacol Rev**. 57**,** 253-277, 2005.

PAINTER, E.P. The chemistry and toxicity of selenium compounds, with special reference to the selenium problem. **Chem Rev.** 28, 179–213, 1941.

PARNHAM, M., SIES, H. Ebselen: prospective therapy for cerebral ischaemia. **Expert Opin Investig Drugs**. 9**,** 607-619, 2000.

PARNHAM, M.J., KINDT, S. A novel biologically active seleno-organic compound--III. Effects of PZ 51 (Ebselen) on glutathione peroxidase and secretory activities of mouse macrophages. **Biochem Pharmacol**. 33**,** 3247-3250, 1984.

PARNHAM, M.J., GRAF, E. Pharmacology of synthetic organic selenium compounds. **Prog Drug Res**. 36**,** 9-47, 1991.

PAZ, M.M., RAMOS, M., RAMIREZ, G., SOUZA, D. Differential effects of guanine nucleotides on kainic acid binding and on adenylate cyclase activity in chick optic tectum. **FEBS Lett**. 355**,** 205-208, 1994.

PINES, G., DANBOLT, N.C., BJORAS, M., ZHANG, Y., BENDAHAN, A., EIDE, L., KOEPSELL, H., STORM-MATHISEN, J., SEEBERG, E., KANNER, B.I. Cloning and expression of a rat brain L-glutamate transporter. **Nature**. 360**,** 464-467, 1992.

PORCIUNCULA, L.O., ROCHA, J.B., TAVARES, R.G., GHISLENI, G., REIS, M., SOUZA, D.O. Methylmercury inhibits glutamate uptake by synaptic vesicles from rat brain. **Neuroreport**. 14**,** 577-580, 2003.

Prigol, M.; Wilhelm, E. A.; Schneider, C. C.; Rocha, J. B. T.; Nogueira, C. W.; Zeni, G.; Brain Res. 2007, 147, 226.

PROHASKA, J.R., GANTHER, H.E. Interactions between selenium and methylmercury in rat brain. **Chem Biol Interact**. 16**,** 155-167, 1977.

PUNTEL, R.L., ROOS, D.H., FOLMER, V., NOGUEIRA, C.W., GALINA, A., ASCHNER, M., ROCHA, J.B. Mitochondrial dysfunction induced by different organochalchogens is mediated by thiol oxidation and is not dependent of the classical mitochondrial permeability transition pore opening. **Toxicol Sci**. 117**,** 133-143, 2010.

RAMOS, M., SOUZA, D.O., RAMIREZ, G. Specific binding of [3H]GppNHp to extracellular membrane receptors in chick cerebellum: possible involvement of kainic acid receptors. **FEBS Lett**. 406**,** 114-118, 1997.

RATHBONE, M.P., MIDDLEMISS, P.J., GYSBERS, J.W., ANDREW, C., HERMAN, M.A., REED, J.K., CICCARELLI, R., DI IORIO, P., CACIAGLI, F. Trophic effects of purines in neurons and glial cells. **Prog Neurobiol**. 59**,** 663-690, 1999.

RICE, D.C. Evidence for delayed neurotoxicity produced by methylmercury. **Neurotoxicology**. 17**,** 583-596, 1996.

ROBINSON, M.B. The family of sodium-dependent glutamate transporters: a focus on the GLT-1/EAAT2 subtype. **Neurochem Int**. 33**,** 479-491, 1998.

ROCHA, J.T., SPERANÇA, A., NOGUEIRA, C.W., ZENI, G. Hypolipidaemic activity of orally administered diphenyl diselenide in Triton WR-1339-induced hyperlipidaemia in mice. **J Pharm Pharmacol.** 61, 1673-1679, 2009.

ROCK, D.M., MACDONALD, R.L. The polyamine spermine has multiple actions on N-methyl-D-aspartate receptor single-channel currents in cultured cortical neurons. **Mol Pharmacol**. 41**,** 83-88, 1992.

ROESLER, R., VIANNA, M.R., LARA, D.R., IZQUIERDO, I., SCHMIDT, A.P., SOUZA, D.O. Guanosine impairs inhibitory avoidance performance in rats. **Neuroreport**. 11**,** 2537-2540, 2000.

ROOS, D.H., PUNTEL, R.L., FARINA, M., ASCHNER, M., BOHRER, D., ROCHA, J.B., DE VARGAS BARBOSA, N.B. Modulation of methylmercury uptake by methionine: prevention of mitochondrial dysfunction in rat liver slices by a mimicry mechanism. **Toxicol Appl Pharmacol**. 252**,** 28-35, 2011.

ROSA, R.M., FLORES, D.G., APPELT, H.R., BRAGA, A.L., HENRIQUES, J.A.P., ROESLER, R. Facilitation of long-term object recognition memory by pretraining administration of diphenyl diselenide in mice. **Neurosci Lett.** 341, 217-220, 2003.

ROTHSTEIN, J.D., DYKES-HOBERG, M., PARDO, C.A., BRISTOL, L.A., JIN, L., KUNCL, R.W., KANAI, Y., HEDIGER, M.A., WANG, Y., SCHIELKE, J.P., WELTY, D.F. Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. **Neuron**. 16**,** 675-686, 1996.

ROTRUCK, J.T., POPE, A.L., GANTHER, H.E., SWANSON, A.B., HAFEMAN, D.G., HOEKSTRA, W.G. Selenium: biochemical role as a component of glutathione peroxidase. **Science**. 179**,** 588-590, 1973.

SAIJOH, K., FUKUNAGA, T., KATSUYAMA, H., LEE, M.J., SUMINO, K. Effects of methylmercury on protein kinase A and protein kinase C in the mouse brain. **Environ Res**. 63**,** 264-273, 1993.

SAITO, I., ASANO, T., SANO, K., TAKAKURA, K., ABE, H., YOSHIMOTO, T., KIKUCHI, H., OHTA, T., ISHIBASHI, S. Neuroprotective effect of an antioxidant, ebselen, in patients with delayed neurological deficits after aneurysmal subarachnoid hemorrhage. **Neurosurgery**. 42**,** 269-277; discussion 277-268, 1998.

SANDALOVA, T., ZHONG, L., LINDQVIST, Y., HOLMGREN, A., SCHNEIDER, G. Three-dimensional structure of a mammalian thioredoxin reductase: implications for mechanism and evolution of a selenocysteine-dependent enzyme. **Proc Natl Acad Sci U S A**. 98**,** 9533-9538, 2001.

SANFELIU, C., SEBASTIA, J., KI, S.U. Methylmercury neurotoxicity in cultures of human neurons, astrocytes, neuroblastoma cells. **Neurotoxicology**. 22**,** 317-327, 2001.

SARAFIAN, T.A., VERITY, M.A. Oxidative mechanisms underlying methyl mercury neurotoxicity, **Int J Dev Neurosci.** 9, 147–153. 1991.

SARAFIAN, T.A. Methyl mercury increases intracellular Ca2+ and inositol phosphate levels in cultured cerebellar granule neurons. **J Neurochem**. 61**,** 648-657, 1993.

SARAFIAN, T.A. Methylmercury-induced generation of free radicals: biological implications. **Met Ions Biol Syst**. 36**,** 415-444, 1999.

SATTLER, R., TYMIANSKI, M. Molecular mechanisms of calcium-dependent excitotoxicity. **J Mol Med (Berl)**. 78**,** 3-13, 2000.

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., 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, 2008a.

STANGHERLIN, E.C., LUCHESE, C., PINTON, S., ROCHA, J.B.T., NOGUEIRA, C.W., Sub-chronical exposure to diphenyl diselenide enhances acquisition and retention of spatial memory in rats. **Brain Res.** 1201, 106-113, 2008b.

SCHWARZ, K., FOLTZ, C.M. Selenium is an integral part of factor-3 against dietary necrotic liver degeneration. **J Am Chem Soc.** 79, 3292–3293, 1957.

SCHIONNING, J.D., EIDE, R., ERNST, E., DANSCHER, G., MOLLER-MADSEN, B. The effect of selenium on the localization of autometallographic mercury in dorsal root ganglia of rats. **Histochem J**. 29**,** 183-191, 1997.

SCHIONNING, J.D. Experimental neurotoxicity of mercury. Autometallographic and stereologic studies on rat dorsal root ganglion and spinal cord. **APMIS Suppl**. 99**,** 1-32, 2000.

SCHMIDT, A.P., LARA, D.R., DE FARIA MARASCHIN, J., DA SILVEIRA PERLA, A., ONOFRE SOUZA, D. Guanosine and GMP prevent seizures induced by quinolinic acid in mice. **Brain Res**. 864**,** 40-43, 2000.

SCHMIDT, A.P., AVILA, T.T., SOUZA, D.O. Intracerebroventricular guanine-based purines protect against seizures induced by quinolinic acid in mice. **Neurochem Res**. 30**,** 69-73, 2005.

SCHMIDT, A.P., LARA, D.R., SOUZA, D.O. Proposal of a guanine-based purinergic system in the mammalian central nervous system. **Pharmacol Ther**. 116**,** 401-416, 2007.

SCHULZ, J.B., HENSHAW, D.R., SIWEK, D., JENKINS, B.G., FERRANTE, R.J., CIPOLLONI, P.B., KOWALL, N.W., ROSEN, B.R., BEAL, M.F. Involvement of free radicals in excitotoxicity in vivo. **J Neurochem**. 64**,** 2239-2247, 1995.

SEO, J.Y., LEE, C.H., CHO, J.H., CHOI, J.H., YOO, K.Y., KIM, D.W., PARK, O.K., LI, H., CHOI, S.Y., HWANG, I.K., WON, M.H. Neuroprotection of ebselen against ischemia/reperfusion injury involves GABA shunt enzymes. **J Neurol Sci**. 285**,** 88-94, 2009.

SHANKER, G., ASCHNER, M. Identification and characterization of uptake systems for cystine and cysteine in cultured astrocytes and neurons: evidence for methylmercury-targeted disruption of astrocyte transport. **J Neurosci Res**. 66**,** 998-1002, 2001.

SHANKER, G., SYVERSEN, T., ASCHNER, J.L., ASCHNER, M. Modulatory effect of glutathione status and antioxidants on methylmercury-induced free radical formation in primary cultures of cerebral astrocytes. **Brain Res Mol Brain Res**. 137**,** 11-22, 2005.

SHENKER, B.J., GUO, T.L., O, I., SHAPIRO, I.M. Induction of apoptosis in human T-cells by methyl mercury: temporal relationship between mitochondrial dysfunction and loss of reductive reserve. **Toxicol Appl Pharmacol**. 157**,** 23-35, 1999.

SIMEONE, T.A., SANCHEZ, R.M., RHO, J.M. Molecular biology and ontogeny of glutamate receptors in the mammalian central nervous system. **J Child Neurol**. 19**,** 343-360; discussion 361, 2004.

SKERFVING, S. Interaction between selenium and methylmercury. **Environ Health Perspect**. 25**,** 57-65, 1978.

SOARES, F.A., SCHMIDT, A.P., FARINA, M., FRIZZO, M.E., TAVARES, R.G., PORTELA, L.V., LARA, D.R., SOUZA, D.O. Anticonvulsant effect of GMP depends on its conversion to guanosine. **Brain Res**. 1005**,** 182-186, 2004.

SOMMER, B., SEEBURG, P.H. Glutamate receptor channels: novel properties and new clones. **Trends Pharmacol Sci**. 13**,** 291-296, 1992.

SOUZA, D.O., RAMIREZ, G. Effects of guanine nucleotides on kainic acid binding and on adenylate cyclase in chick optic tectum and cerebellum. **J Mol Neurosci**. 3**,** 39-45, 1991.

STORCK, T., SCHULTE, S., HOFMANN, K., STOFFEL, W. Structure, expression, and functional analysis of a Na(+)-dependent glutamate/aspartate transporter from rat brain. **Proc Natl Acad Sci U S A**. 89**,** 10955-10959, 1992.

SUGIURA, Y., HOJO, Y., TAMAI, Y., TANAKA, H. Letter: Selenium protection against mercury toxicity. Binding of methylmercury by the selenohydryl-containing ligand. **J Am Chem Soc**. 98**,** 2339-2341, 1976.

SZATKOWSKI, M., BARBOUR, B., ATTWELL, D. Non-vesicular release of glutamate from glial cells by reversed electrogenic glutamate uptake. **Nature**. 348**,** 443-446, 1990.

TAKASAGO, T., PETERS, E.E., GRAHAM, D.I., MASAYASU, H., MACRAE, I.M. Neuroprotective efficacy of ebselen, an anti-oxidant with anti-inflammatory actions, in a rodent model of permanent middle cerebral artery occlusion. **Br J Pharmacol**. 122**,** 1251-1256, 1997.

TAKEUCHI, T., MORIKAWA, N., MATSUMOTO, H., SHIRAISHI, Y. A pathological study of Minamata disease in Japan. **Acta Neuropathol.** 2, 40–57, 1962.

TANAKA, K., WATASE, K., MANABE, T., YAMADA, K., WATANABE, M., TAKAHASHI, K., IWAMA, H., NISHIKAWA, T., ICHIHARA, N., KIKUCHI, T., OKUYAMA, S., KAWASHIMA, N., HORI, S., TAKIMOTO, M., WADA, K. Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. **Science**. 276**,** 1699-1702, 1997.

TANG, C.M., DICHTER, M., MORAD, M. Quisqualate activates a rapidly inactivating high conductance ionic channel in hippocampal neurons. **Science**. 243**,** 1474-1477, 1989.

TASCA, C.I., SANTOS, T.G., TAVARES, R.G., BATTASTINI, A.M., ROCHA, J.B., SOUZA, D.O. Guanine derivatives modulate L-glutamate uptake into rat brain synaptic vesicles. **Neurochem Int**. 44**,** 423-431, 2004.

TAYLOR, T.J., RIEDESS, F., KOESS, J.J. The role of Hg2+ and methylmercury in lipid peroxidation. **Fed Proc.** 32, 261, 1973.

TROTTI, D., NUSSBERGER, S., VOLTERRA, A., HEDIGER, M.A. Differential modulation of the uptake currents by redox interconversion of cysteine residues in the human neuronal glutamate transporter EAAC1. **Eur J Neurosci**. 9**,** 2207-2212, 1997a.

TROTTI, D., RIZZINI, B.L., ROSSI, D., HAUGETO, O., RACAGNI, G., DANBOLT, N.C., VOLTERRA, A. Neuronal and glial glutamate transporters possess an SH-based redox regulatory mechanism. **Eur J Neurosci**. 9**,** 1236-1243, 1997b.

TROTTI, D., DANBOLT, N.C., VOLTERRA, A. Glutamate transporters are oxidant-vulnerable: a molecular link between oxidative and excitotoxic neurodegeneration? **Trends Pharmacol Sci**. 19**,** 328-334, 1998.

TRUSSELL, L.O., FISCHBACH, G.D. Glutamate receptor desensitization and its role in synaptic transmission. **Neuron**. 3**,** 209-218, 1989.

TUNC, T., UYSAL, B., ATABEK, C., KESIK, V., CALISKAN, B., OZTAS, E., ERSOZ, N., OTER, S., GUVEN, A. Erdosteine and ebselen as useful agents in intestinal ischemia/reperfusion injury. **J Surg Res**. 155**,** 210-216, 2009.

VALKO, M., MORRIS, H., CRONIN, M.T. Metals, toxicity and oxidative stress. **Curr Med Chem**. 12**,** 1161-1208, 2005.

VINADE, E.R., SCHMIDT, A.P., FRIZZO, M.E., PORTELA, L.V., SOARES, F.A., SCHWALM, F.D., ELISABETSKY, E., IZQUIERDO, I., SOUZA, D.O. Effects of chronic administered guanosine on behavioral parameters and brain glutamate uptake in rats. **J Neurosci Res**. 79**,** 248-253, 2005.

VOLTERRA, A., TROTTI, D., RACAGNI, G. Glutamate uptake is inhibited by arachidonic acid and oxygen radicals via two distinct and additive mechanisms. **Mol Pharmacol**. 46**,** 986-992, 1994.

WADICHE, J.I., AMARA, S.G., KAVANAUGH, M.P. Ion fluxes associated with excitatory amino acid transport. **Neuron**. 15**,** 721-728, 1995.

WAGNER, C., SUDATI, J.H., NOGUEIRA, C.W., ROCHA, J.B. In vivo and in vitro inhibition of mice thioredoxin reductase by methylmercury. **Biometals**. 23**,** 1171-1177, 2010a.

WAGNER, C., VARGAS, A.P., ROOS, D.H., MOREL, A.F., FARINA, M., NOGUEIRA, C.W., ASCHNER, M., ROCHA, J.B. Comparative study of quercetin and its two glycoside derivatives quercitrin and rutin against methylmercury (MeHg)-induced ROS production in rat brain slices. **Arch Toxicol**. 84**,** 89-97, 2010b.

WALTON, H.S., DODD, P.R. Glutamate-glutamine cycling in Alzheimer's disease. **Neurochem Int**. 50**,** 1052-1066, 2007.

WENDEL, A., FAUSEL, M., SAFAYHI, H., TIEGS, G., OTTER, R. A novel biologically active seleno-organic compound--II. Activity of PZ 51 in relation to glutathione peroxidase. **Biochem Pharmacol**. 33**,** 3241-3245, 1984.

WHO. Environmental Health Criteria 1. Mercury. International Program on Chemical Safety. Geneva: World Health Organization, 1976.

WHO. Environmental Health Criteria 101: Methylmercury. International Program on Chemical Safety. Geneva:World Health Organization, 1990.

WILSON, S.R., ZUCKER, P.A., HUANG, R-R.C. Spector, A. A development of synthetic compounds with glutathione peroxidase activity. **J Am Chem Soc.** 111, 5936–5939, 1989.

XIONG, S., MARKESBERY, W.R., SHAO, C., LOVELL, M.A. Seleno-L-methionine protects against beta-amyloid and iron/hydrogen peroxide-mediated neuron death. **Antioxid Redox Signal**. 9**,** 457-467, 2007.

YAMAGUCHI, T., SANO, K., TAKAKURA, K., SAITO, I., SHINOHARA, Y., ASANO, T., YASUHARA, H. Ebselen in acute ischemic stroke: a placebo-controlled, double-blind clinical trial. Ebselen Study Group. **Stroke**. 29**,** 12-17, 1998.

YANG, C.F., SHEN, H.M., ONG, C.N. Intracellular thiol depletion causes mitochondrial permeability transition in ebselen-induced apoptosis. **Arch Biochem Biophys**. 380**,** 319-330, 2000a.

YANG, C.F., SHEN, H.M., ONG, C.N. Ebselen induces apoptosis in HepG(2) cells through rapid depletion of intracellular thiols. **Arch Biochem Biophys**. 374**,** 142-152, 2000b.

YEE, S., CHOI, B.H. Methylmercury poisoning induces oxidative stress in the mouse brain. **Exp Mol Pathol**. 60**,** 188-196, 1994.

YEE, S., CHOI, B.H. Oxidative stress in neurotoxic effects of methylmercury poisoning. **Neurotoxicology**. 17**,** 17-26, 1996.

YIN, Z., LEE, E., NI, M., JIANG, H., MILATOVIC, D., RONGZHU, L., FARINA, M., ROCHA, J.B., ASCHNER, M. Methylmercury-induced alterations in astrocyte functions are attenuated by ebselen. **Neurotoxicology**. 32**,** 291-299, 2011.

YONAHA, M., SAITO, M., SAGAI, M. Stimulation of lipid peroxidation by methylmercury in rats. **Life Sci.** 32, 1507–1514, 1983.

YOSHINO, Y., MOZAI, T., NAKAO, K. Biochemical changes in the brain in rats poisoned with an alkymercury compound, with special reference to the inhibition of protein synthesis in brain cortex slices. **J Neurochem**. 13**,** 1223-1230, 1966.

ZALUPS, R.K. Molecular interactions with mercury in the kidney. **Pharmacol Rev**. 52**,** 113-143, 2000.

ZAUNER, A., BULLOCK, R. The role of excitatory amino acids in severe brain trauma: opportunities for therapy: a review. **J Neurotrauma**. 12**,** 547-554, 1995.

ZERANGUE, N., KAVANAUGH, M.P. Flux coupling in a neuronal glutamate transporter. **Nature**. 383**,** 634-637, 1996.

ZHAO, R., HOLMGREN, A. A novel antioxidant mechanism of ebselen involving ebselen diselenide, a substrate of mammalian thioredoxin and thioredoxin reductase. **J Biol Chem**. 277**,** 39456-39462, 2002.

ZHAO, R., MASAYASU, H., HOLMGREN, A. Ebselen: a substrate for human thioredoxin reductase strongly stimulating its hydroperoxide reductase activity and a superfast thioredoxin oxidant. **Proc Natl Acad Sci U S A**. 99**,** 8579-8584, 2002.

ZHONG, L.W., ARNER, E.S.J., HOLMGREN, A. Structure and mechanism of mammalian thioredoxin reductase: The active site is a redox-active selenolthiol/selenenylsulfide formed from the conserved cysteine-selenocysteine sequence. **Proc Natl Acad Sci U S A.** 97**,** 5854-5859, 2000.