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ATIVIDADES HEPATO E NEUROPROTETORA DO DIETIL-2-FENIL-2-TELUROFENIL VINILFOSFONATO

TESE DE DOUTORADO

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ATIVIDADES HEPATO E NEUROPROTETORA DO DIETIL-2-FENIL-2-TELUROFENIL VINILFOSFONATO

por

Daiana Silva de Ávila

Tese apresentada ao Curso de pós - graduação em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS) como requisito para obtenção do grau de **Doutor em Bioquímica Toxicológica.**

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ATIVIDADES NEURO E HEPATOPROTETORA DO DIETIL-2-FENIL-2-TELUROFENIL VINILFOSFONATO

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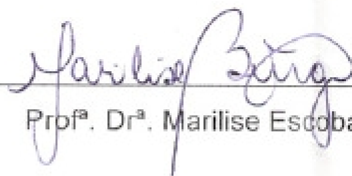

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Toda a nossa ciência, comparada com a realidade, é primitiva e infantil - e, no entanto, é a coisa mais preciosa que temos.

Albert Einstein

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Resumo

Tese de doutorado
Programa de pós-graduação em Bioquímica Toxicológica
Universidade Federal de Santa Maria

ATIVIDADES HEPATO E NEUROPROTETORA DO DIETIL-2-FENIL-2-TELUROFENIL VINILFOSFONATO
AUTORA: DAIANA SILVA DE ÁVILA
ORIENTADOR: FÉLIX ALEXANDRE ANTUNES SOARES
CO-ORIENTADOR: JOÃO BATISTA TEIXEIRA DA ROCHA
Local e data da defesa: Santa Maria, 27 de março de 2009.

Apesar do crescente uso dos compostos orgânicos de telúrio na química e na bioquímica, pouco se sabe sobre a farmacologia de tais compostos. O Dietil-2-fenil-2-telurofenil vinilfosfonato (DPTVP) é um telureto vinílico que apresenta baixa toxicidade em camundongos e uma elevada atividade antioxidante in vitro. Estes dados prévios da literatura nos encorajaram a avaliar mais a fundo esse potencial antioxidante e os benefícios farmacológicos que o composto poderia proporcionar. Foi verificado que o composto protege contra o aumento na peroxidação lipídica em cérebro induzida por agentes neurotóxicos como o ácido quinolínico (QA) e o nitroprussiato de sódio (SNP) in vitro em baixas concentrações (a partir de 1µM). O DPTVP também protegeu contra a disfunção mitocondrial induzida por SNP em córtex, hipocampo e estriado de ratos. Além disso, o composto demonstrou baixa neurotoxicidade, uma vez que não alterou a viabilidade mitocondrial nem o sistema glutamatérgico *in vitro* nas concentrações antioxidantes (até 50µM). Diante da possível atividade neurotoprotetora observada in vitro pelo DPTVP, o modelo de neurotoxicidade induzido por Mn foi utilizado a fim de avaliá-lo in vivo e ex vivo. A exposição ao Mn por 4 meses (137mg/Kg) causou um prejuízo à atividade exploratória e motora dos ratos, bem como alterações em parâmetros bioquímicos analizados como aumento na lipoperoxidação, redução da viabilidade mitocondrial e redução na captação de $[^3H]$glutamato no estriado, aliado ao aumento nos níveis do metal nessa estrutura. O tratamento por duas semanas com o DPTVP recuperou parcialmente as alterações neurocomportamentais devido à reversão dos danos oxidativos causados pelo Mn no estriado observados post mortem. Além disso, os animais tratados com o DPTVP não demonstraram níveis elevados de Mn no estriado, indicando que o composto pode alterar as condições de transporte do Mn nesse area. Também foi verificada a atividade hepatoprotetora do DPTVP contra o acetaminofeno (APAP) em camundongos. Ambas as doses (200 e 300mg/Kg de APAP) causaram alterações hepáticas como depleção de SH não-protéico, aumento nos níveis de TBARS, inibição da δ-ALA-D, extravazamento de ALT para o sangue e danos morfológicos aos hepatócitos, entretanto em diferentes intensidades. Dessa maneira, o tratamento com DPTVP (30, 50 e 100µmol/Kg) foi bastante efetivo quando a dose de APAP foi a de 200mg/Kg. Já na dose de 300mg/Kg de APAP, o composto apenas recuperou de fato as alterações histomorfológicas dos hepatócitos. O presente trabalho também evidenciou que a atividade antioxidante do telureto vinílico provavelmente deve-se à sua atividade neutralizadora ou "scavenger",

uma vez que o composto mostrou-se efetivo em capturar tanto espécies reativas de oxigênio (ERO) quanto de nitrogênio (ERN), como o H_2O_2, o OH•, ON• e o $ONOO^-$, provavelmente devido à formação de teluróxido favorecida pela estrurura do composto. Esses resultados sugerem que o DPTVP possui ações hepato e neuroprotetoras em baixas doses devido ao seu potencial antioxidante, e que a formação de teluróxido seja essencial para esses efeitos.

Palavras-chave: telúrio, glutamato, manganês, neuroprotetor, acetaminofeno, hepatoprotetor, antioxidante.

Abstract

PhD`s thesis
Graduation Program of Toxicologial Biochemistry
Federal University of Santa Maria

HEPATO AND NEUROPROTECTIVE ACTIVITIES OF DIETHYL-2-PHENYL-2-TELLUROPHENYL VINYLPHOSPHONATE

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Date and place of the defense: Santa Maria, March 27th, 2009.

Despite the growing use of organotellurium compounds in the chemistry and biochemistry field, little is known about the pharmacology of these compounds. The diethyl-2-phenyl-2-tellurophenyl vinylphosphonate (DPTVP) is a vinylic telluride wich shows low toxicity in mice and a high antioxidant activity in vitro. These previous data encoraged us to evaluate the antioxidant potential and the pharmacological benefits that the compound could provide. It was observed that the compound can protect at low concentrations (from 1µM) against the increased lipid peroxidation in brain induced by the neurotoxic agents sodium nitropruside (SNP) and quinolinic acid (QA) in vitro. Furthermore, DPTVP also protected against the mitochondrial dysfunction induced by SNP in cortex, hippocampus and striatum of rats. Besides, the compound did not show neurotoxic effect, once it did not altered mitochondrial viability and neither the glutamatergic system in vitro at the antioxidant concentrations (until 50µM). Considering the possible neuroprotective effect of DPTVP in vitro, the model of Mn neurotoxicity in rats was used in order to evaluate it in vivo and ex vivo. The exposure to Mn for 4 months (137mg/Kg) caused an impairment of the exploratory and motor activity of the rats, as well as alterations in the biochemical parameter analyzed, such as increase in the lipid peroxidation and reduction in the mitochondrial viability and in the [^{3}H] glutamate uptake in striatum, allied to increase in the Mn levels in this brain structure. The treatment for two weeks with DPTVP partially recoved from the neurobehavioral alterations, probably due to the protective effect against the oxidative damage caused by Mn in the striatum observed post mortem. Nevertheless, the animals treated with DPTVP did not showed increased levels of Mn in their striatum, indicating that the compound can act not only as an antioxidant against manganism, but can also impairs the influx or facilitates Mn efflux in thia area. The hepatoprotective activity of DPTVP was also verified against the acetaminophen (APAP) in mice. Both APAP does (200 and 300mg/Kg) caused hepatic alterations sucs as non-proteic SH depletion, Increase in TBARS levels, inhibition of δ- ALA D, ALT leakage and morphologic damage to the hepatocytes, nevertheless at different intensities. Hence, The treatment with DPTVP (30, 50 and 100µmol/Kg) showed effectiveness when the APAP dose pre-administeres was 200mg/Kg. Against the dose of 300mg/Kg, the compound has just recovered from the histomorphologic alterations to hepatocytes. The present estudy has also evidenced that the antioxidant activity depicted by the vinilc telluride is due to its scavenger activity, once the compound was able to neutralize

reative oxygen (ROS) and reactive nitrogen species (RNS), such as H_2O_2, OH•, ON• and $ONOO^-$, probably due to the formation of telluroxide, which is due to the molecular structure of the compound. Taken together, these results suggest that DPTVP has neuro and hepatoprotective actions at low doses probably due to its strong antioxidant activity and that the formation of telluroxide is very important to these effects.

Key Words: tellurium, glutamate, manganese, neuroprotector, acetaminophen, hepatoprotector, antioxidant.

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LISTA DE ABREVIATURAS

AINES: antiinflamatórios não- esteroidais

ALT: alanina aminotransferase

APAP: acetaminofeno

AS-101: telurato de tricloro amônio-dioxoetileno-O,O

AST: aspartato aminotransferase

ATP: adenosina trifosfato

Ca: cálcio

CAT: catalase

CB: receptores canabinóides

CYP: sistema do citocromo P450

DA: dopamina

DDR: dose diária recomendada

DMT: transportador de metal divalente

DNA: ácido desoxirribonucléico

DPPH: 2,2-difenil-1-picrilidrazila

DPTVP: dietil-2-fenil-2-telurofenil vinilfosfonato

DTT: ditiotreitol

EAAT: transportadores de aminoácidos excitatórios

ER: espécie reativa

ERN: espécies reativas de nitrogênio

ERO: espécies reactivas de oxigênio

Fe: ferro

GLAST: transportador de glutamato/aspartato

GLT-1: transportador de glutamato tipo 1

GPX: glutationa peroxidase

GSH: glutationa reduzida

GSSG: glutationa oxidada

H_2O_2: peróxido de hidrogênio

i.p.: intraperitoneal

IC_{50}: concentração initória 50%

K: potássio

LD50: dose letal 50%

LDH: lactato desidrogenase

LP: lipoperoxidação

MDA: malondialdeído

mGluRs: receptores metabotrópicos de glutamato

Mn: manganês

MTT: brometo de 3-(4,5-dimetilltiazol-2-il)-2,5-difeniltetrazolium

Na^+/K^+ ATPase: sódio/potássio ATPase

NAC: N-acetilcisteína

NAPQI: N-acetil-p- benzoquinonaimina

NADPH: nicotinamida adenina dinucleotídeo fosfato

NMDA: N-metil-D-aspartato

NOS: óxido nítrico sintase

$O_2\bullet^-$: radical ânion superóxido

$OH^\cdot$: radical hidroxil

ON•: radical óxido nítrico

$ONOO^-$: peroxinitrito

PG: prostaglandinas

QA: ácido quinolínico

$R^\cdot$: radical de fosfolipídeo

RH: ácido graxo insaturado

RL: radical livre

$RO^\cdot$: radical alcoxil

$ROO^\cdot$: radical peroxil

ROOH: hidroperóxido lipídico

s.c.: subcutâneo

-SH: grupamento tiólico

SNC: sistema nervoso central

SNP: nitroprussiato de sódio

SOD: superóxido dismutase

S-S: dissulfeto

RNAm: ácido ribonucléico mitocondrial

TBARS: subtancias reativas ao acido tiobarbitúrico

Te: telúrio

TNF-α: fator de necrose tumoral

TRXr: tiorredoxina redutase

TRX-SH: tiorredoxina reduzida

VGLU: transportadores de glutamato vesicular

δ- ALA-D: delta- aminolevulinato desidratase

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APRESENTAÇÃO

No item I, **INTRODUÇÃO**, está descrita uma sucinta introdução sobre a tese.

No item II, **REVISÃO BIBLIOGRÁFICA**, sobre os temas trabalhados nesta dissertação.

Os **OBJETIVOS**, geral e específicos, estão organizados no item III.

Os **RESULTADOS** estão dispostos na forma de artigos, um já publicado e outros três na forma de manuscritos submetidos à publicação, organizados no item IV. Os artigos científicos estão descritos na íntegra, contendo as seções introdução, materiais e métodos, resultados, discussão e referências bibliográficas.

No item V, **DISCUSSÃO**, estão apresentados as interpretações e comentários gerais sobre os artigos científicos aqui apresentados. Também estão dispostos alguns pontos de vista da autora em relação aos resultados obtidos.

No item VI, **CONCLUSÕES**, são apresentadas as conclusões gerais do presente trabalho.

No item VII, **PERSPECTIVAS**, estão expostos alguns possíveis estudos para a continuação deste trabalho.

O item VIII, **REFERÊNCIAS BIBLIOGRÁFICAS**, refere-se somente às citações que aparecem nos itens I e V (Introdução e Discussão).

I. Introdução

Os compostos orgânicos de Telúrio vêm sendo estudados por diversos pesquisadores quanto às suas propriedades tóxicas e farmacológicas. Tais compostos são descritos como potentes antioxidantes e quimioprotetores (Sredni *et al.*, 1987; Engman *et al.*, 1995; Briviba *et al.*, 1998; Engman *et al.*, 2000), entretanto eles apresentam certa toxicidade, principalmente devido à capacidade de oxidarem grupamentos SH de moléculas biologicamente importantes (Toews *et al.*, 1997; Laden e Porter, 2001; Nogueira *et al.*, 2003; Borges *et al.*, 2005).

Entretanto, uma série de novos compostos pertencente à classe dos teluretos viníIcos têm apresentado resultados bastante promissores, uma vez que possuem baixa toxicidade e boa atividade antioxidante (Savegnago *et al.*, 2006; Borges *et al.*, 2008). Dentre estes compostos destaca-se o dietil-2-fenil-2-telurofenil vinilfosfonato (DPTVP), alvo de estudo nessa tese.

O estresse oxidativo é definido como um desequilíbrio entre a produção de espécies reativas de oxigênio (ERO) e/ou nitorgênio (ERN) e o sistema antioxidante celular (Halliwell e Gutterridge, 2003). Tais espécies são produzidas em excesso durante alguns processos patológicos, causando alterações celulares como oxidação de proteínas e lipídeos, disfunção mitocondrial, dano ao DNA podendo culminar com morte celular (Halliwell, 2001). Nesse contexto, diversos estudos têm demonstrado que o tratamento com antioxidantes tem se mostrado bastante eficaz em diversos modelos animais de neuro e hepatotoxicidade (Halliwell, 2001).

A excitotoxicidade induzida por glutamato é um importante modelo de neurotoxicidade, uma vez que diversas doenças neurodegenerativas estão relacionadas com alterações na homeostase desse neurotransmissor (Krieglstein, 1997). Alguns compostos químicos podem mimetizar os danos excitotóxicos causados pelo glutamato, como o nitroprussiato de sódio (SNP) e o ácido quinolínico (QA) (Stone, 1991; Rauhala *et al.*, 1998). Além disso, o modelo de parkinsonismo induzido por manganês (Mn) também envolve alterações glutamatérgicas cujo mecanismo ainda não está bem

esclarecido (Aschner *et al.*, 2007). Além disso, o estresse oxidativo presente na neurotoxicidade induzida por Mn é um fator bastante importante na patogênese da doença (Yin *et al.*, 2008).

O acetaminofeno é um analgésico amplamente utilizado, entretanto em altas doses apresenta efeitos hepatotóxicos graves, devido à depleção de glutationa (GSH), molécula importante no sistema antioxidante celular (Larson, 2007). O estresse oxidativo que se desencadeia acaba causando graves danos histomorfológicos aos hepatócitos, podendo culminar em morte por necrose (Mcjunkin *et al.*, 1976).

Considerando a baixa toxicidade do DPTVP associada à potente atividade in vitro, a falta de estudos farmacológicos com esse composto em modelos animais nos quais o estresse oxidativo está envolvido e a necessidade de se avaliar o mecanismo de ação desse composto, a presente tese tem como finalidade avaliar as possíveis atividades neuro e hepatoprotetora do DPTVP, o envolvimento do seu potencial antioxidante nesses efeitos e sugerir um possível mecanismo de ação.

II. Revisão Bibliográfica

II.1. Telúrio

O elemento telúrio foi descoberto em 1782, pertencendo ao grupo 16 da tabela periódica, denominada família dos calcogênios, assim como o selênio e o enxofre. Pode apresentar-se com diferentes estados de oxidação: Te^{+6} (telurato), Te^{+4} (telurito), Te^{0} (telúrio elementar) e Te^{+2} (telureto) (Scansetti, 1992). É encontrado com maior freqüência na forma de teluretos de ouro, bismuto, chumbo e prata.

O Te^{0} é utilizado no manufaturamento de semicondutores e outros componentes eletrônicos. Ele também é utilizado na produção industrial de vidro e aço e como um aditivo anti-detonante da gasolina (Fairhill, 1969). Além disso, é empregado no processo de síntese de alguns fármacos e explosivos, na vulcanização da borracha, em lubrificantes sólidos e na petroquímica (Taylor, 1996).

Em 1967, Shoereder e cols (Schroeder *et al.*, 1967) determinaram, utilizando espectrometria de absorção atômica, que os organismos humanos possuiriam aproximadamente 600 mg de telúrio, uma quantidade relativamente grande em comparação com outros elementos - traço como o ferro e o zinco. Apesar desta grande quantidade, nenhuma função fisiológica foi ainda atribuída ao telúrio.

II.1.1. Compostos orgânicos de telúrio

O primeiro composto orgânico de telúrio foi sintetizado por Friedrich Wöhler em 1840 (Wohler, 1840). Desde sua descoberta até metade do século 20, a química dos compostos orgânicos de telúrio permaneceu obscura e, devido ao pouco interesse dos pesquisadores, às difíceis condições de síntese destes compostos e, especialmente, pelo mau odor que estes exalavam, poucas são as publicações durante este período. Apenas a partir de 1970 os compostos orgânicos de telúrio começaram a serem explorados pelos químicos orgânicos, refletindo no crescimento exponencial de artigos

publicados desde então (Klaman, 1990). Vários destes compostos, com diferentes características e estruturas químicas, vêm sendo estudados quanto às suas propriedades fármaco- tóxicológicas, sendo alguns deles já reportados na literatura.

II.1.1.1. Toxicologia dos compostos orgânicos de telúrio

Assim como o telúrio elementar e os sais inorgânicos, os compostos orgânicos de telúrio são bastante tóxicos, e a intensidade desta toxicidade depende da estrutura do composto, da dose administrada e do tipo de animal testado (Nogueira, Zeni *et al.*, 2004).

A toxicidade destes compostos deve-se principalmente pela interação com -SH de moléculas biologicamente ativas. Os compostos orgânicos de telúrio têm a capacidade de oxidar estes grupamentos –SH, inativando enzimas e/ou diminuindo a concentração de moléculas sulfidrílicas não- protéicas, como a glutationa (GSH) (Blais *et al.*, 1972; Deuticke *et al.*, 1992).

A oxidação da GSH em GSSG pode ser um dos principais fatores da toxicidade causada pelos compostos orgânicos de telúrio (Barbosa *et al.*, 1998). A GSH é uma importante biomolécula necessária ao sistema antioxidante da glutationa peroxidase. Esta enzima requer GSH para neutralizar o peróxido de hidrogênio em água, gerando GSSG, que retorna a GSH pela ação da glutationa redutase, às custas do equivalente redutor nicotinamida adenina dinucleotídeo fosfato (NADPH). Outras biomoléculas sulfidrílicas como a cisteína, a coenzima A e o ácido diidrolipóico também podem ser alvos dos compostos orgânicos de telúrio, porém não há relatos na literatura sobre estas possibilidades.

A enzima sulfidrílica δ-ALA-D, que catalisa condensação assimétrica de duas moléculas de ácido aminolevulínico em porfobilinogênio, é um dos alvos dos compostos orgânicos de telúrio mais estudado. Esta enzima possui no seu sítio ativo dois resíduos cisteinil, que são facilmente oxidados *in vitro* e *in vivo* por compostos de telúrio orgânicos, levando a sua inibição (Maciel *et al.*, 2000; Meotti *et al.*, 2003; Nogueira, Meotti *et al.*, 2003). Esta inativação impede a continuação da cascata de síntese de

grupamentos heme, importantes na síntese de hemoglobina, citocromos e da enzima catalase. Além disso, esta inibição leva ao acúmulo do substrato da δ-ALA-D, o ácido aminolevulínico, que tem ação pró-oxidante (Bechara *et al.*, 1993; Emanuelli *et al.*, 2001). O mecanismo de inibição pela oxidação dos dois grupamentos –SH desta enzima é evidenciado pela reativação da sua atividade pela adição no meio reacional de ditiotreitol (DTT), um composto dissulfidrílico capaz de doar seus grupamentos –SH à δ-ALA-D, reduzindo as pontes S-S à forma SH, antes oxidada pelos compostos (Nogueira, Zeni *et al.*, 2004).

Maciel et al. (2000) demonstraram a inibição da δ-ALA-D em fígado, cérebro e rim em camundongos após administração aguda (1 dia) de ditelureto de difenila na dose de 500 µmol/Kg, bem como inibição da enzima hepática e cerebral após exposição sub crônica (14 dias) nas doses de 10 e 25µmol/Kg. Meotti et al. (2003) verificaram a inibição de 40% da atividade desta enzima em eritrócitos de camundongos após três dias de administração única de ditelureto de difenila na dose de 150µmol/Kg. Em contraste ao ditelureto de difenila, que inibe fortemente *in vitro* e *in vivo* a δ-ALA-D, um telureto vinílico, o 1-butiltelurenil-2-metiltiohepteno não inibe a atividade da enzima *in vitro*, mas após única administração de 75 µmol/Kg foi capaz de inibi-la em fígado e baço (Savegnago *et al.*, 2006).

As toxicidades hepática e renal *ex vivo* também já foram observadas para alguns compostos orgânicos de telúrio. Os marcadores mais comuns de hepatotoxicidade são as atividades plasmáticas da aspartato aminotransferase (AST) e da alanina aminotransferase (ALT), que se tornam aumentadas quando há dano causado pelos compostos de telúrio (Meotti *et al.*, 2003; Savegnago *et al.*, 2006). A toxicidade renal pode ser evidenciada pela diminuição das funções renais, como diminuição da excreção de creatinina e uréia, que se tornam aumentadas no plasma (Savegnago *et al.*, 2006). Além disso, a Te-fenil-L-telurocisteína, é capaz de depletar GSH e de diminuir a atividade da lactato desidrogenase (LDH) em hepatócitos de ratos tanto quanto o paracetamol, um conhecido agente hepatotóxico (Rooseboom *et al.*, 2002).

A neurotoxicidade causada pelos compostos orgânicos de telúrio é evidenciada por várias alterações nos animais expostos, como por exemplo, alterações na memória, desmielinização, neuropatia periférica (Toews *et al.*, 1997; Goodrum, 1998; Laden e Porter, 2001). A desmielinização e suas conseqüências induzidas por compostos orgânicos de telúrio devem-se à inibição de outra enzima sulfidrílica importante na biossíntese do colesterol, um dos lipídeos presente na mielina, a esqualeno monooxigenase. Esta enzima contém -SH vicinais que são oxidáveis tanto por telúrio elementar (Toews *et al.*, 1991) como pelas formas orgânicas de telúrio, dicloreto de dimetiltelúrio, cloreto de trimetiltelúrio e dimetiltelureto (Goodrum, 1998; Laden e Porter, 2001). Além disso, a Na^+/K^+ ATPase, uma enzima sulfidrílica bastante importante para a manutenção da atividade neuronal normal também é suscetível a agentes oxidantes como os compostos orgânicos de telúrio. Borges et al. (2005) verificaram que o ditelureto de difenila é capaz de inibir esta enzima *in vitro* em baixas concentrações, sendo esta inibição revertida pela co-incubação com DTT, comprovando a oxidação os grupamentos tiólicos (Borges *et al.*, 2005). Além disso, este composto pode também alterar o sistema glutamatérgico, diminuindo a captação vesicular *in vitro* de [^{3}H]glutamato e a união específica de [^{3}H]glutamato no receptor NMDA (N- metil D - aspartato) em preparação de membranas de cérebro *in vitro* e *ex vivo* em baixas concentrações (Nogueira *et al.*, 2001; Nogueira *et al.*, 2002). Isto se deve à presença de grupamentos sulfidrílicos nos transportadores de glutamato, assim como a presença, no receptor NMDA, de um sítio redox dependente da forma reduzida de seus grupos sulfidrílicos para a união específica com o neurotransmissor (Trotti *et al.*, 1997), ambos facilmente oxidáveis pelos compostos orgânicos de telúrio.

Apesar da potencial toxicidade que os compostos orgânicos de telúrio podem exercer sobre os organismos vivos, como foi aqui descrito, as possíveis propriedades farmacológicas terapeuticamente relevantes relatadas na literatura nos encorajam a avaliar novos compostos.

II.1.1.2. Farmacologia dos compostos orgânicos de telúrio

Em 1987, Sredni et al. descreveram pela primeira vez uma atividade farmacológica para um composto orgânico de telúrio, ao demonstrarem as propriedades imunomoduladoras do composto codificado como AS-101 (telurato de tricloro amônio-dioxoetileno-O,O') em camundongos, mediando efeitos antitumorais (Sredni *et al.*, 1987). Além disso, o AS-101 é capaz de estimular células linfóides a produzir citocinas como IL-1, IL-2, TNF-α (Kozenitzky *et al.*, 1992).

A propriedade quimioprotetora dos compostos orgânicos de telúrio, proveniente de seus efeitos citotóxicos, começou a ser explorada por diversos grupos de pesquisa. Apesar da habilidade dos compostos de induzirem apoptose e alterações de outras naturezas a fim de causarem morte celular, um novo alvo para a pesquisa anticâncer vem surgindo, a enzima tiorredoxina redutase (TRXr). Esta enzima, responsável por fornecer equivalentes redutores (tiorredoxina - TRX-SH) às redutases de ribonucleotídeos, têm sua expressão gênica aumentada nas células tumorais, auxiliando no crescimento celular (Grogan *et al.*, 2000). Engman et al. (2003) sintetizaram uma série de compostos análogos do telureto de difenila e observaram que alguns eram bons inibidores da tiorredoxina redutase e inibidores do crescimento de células cancerosas (Engman *et al.*, 2003). Compostos orgânicos de telúrio na forma de sais, solúveis em água, também foram avaliados, e mostraram-se os melhores inibidores da TRXr já testados (Engman *et al.*, 2003). Apesar da hidrofilicidade dos compostos ter restringido a captação celular, foram bastante eficientes contra culturas de câncer de cólon (Engman *et al.*, 2000).

Os teluritos orgânicos também vêm sendo estudados quanto às suas possíveis propriedades antitumorais, sendo o alvo a enzima Catepsina B. Esta enzima é uma protease que auxilia na invasão do tumor, por degradar componentes exracelulares (Buck *et al.*, 1992). Ela possui no seu sítio ativo um resíduo tiólico, cuja oxidação pelos teluritos leva à inativação da enzima, sugerindo uma provável ação anti-metastática destes compostos (Cunha *et al.*, 2005)

Além destas importantes propriedades, alguns compostos orgânicos de telúrio possuem outra característica: são capazes de mimetizar a atividade da glutationa-peroxidase (GPX) que, à custa de GSH, neutraliza o peróxido de hidrogênio (H_2O_2).

Kanda et al. (1999) demonstraram, utilizando a técnica de ressonância magnética nuclear, que vários diaril diteluretos possuem atividade tiol peroxidase, propondo o mecanismo da reação para estes compostos (Kanda *et al.*, 1999). Posteriormente, (Andersson *et al.*, 1993) reportaram a atividade mimética da GPX de diaril teluretos, substituídos com moléculas doadoras de elétrons. Além disso, estes autores apontaram que o efeito antioxidante destes compostos se devia à formação de telurόxido, resultante da oxidação do Te^{+2} à Te^{+4}, que seria novamente reduzido ao estado inicial pela GSH, num mecanismo catalítico, neutralizando radicais livres e as espécies reativas de oxigênio/nitrogênio (Figura 1) (Andersson *et al.*, 1994; Engman *et al.*, 1994)

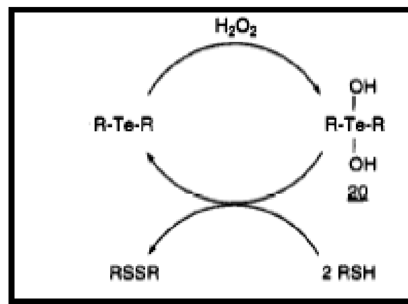


Figura 1: Mecanismo catalítico da atividade glutationa-peroxidase mimética de compostos diarílicos e dialquílicos de telúrio (Andersson et al., 1994).

Briviba et al. (1998) avaliaram alguns compostos orgânicos de telúrio num modelo de dano oxidativo por espécies reativas de nitrogênio, mais especificamente o peroxinitrito ($ONOO^-$) (Briviba *et al.*, 1998). Os autores demonstraram a capacidade dos compostos de neutralizarem o ($ONOO^-$) e inibirem a sua capacidade de nitrosilar proteínas, também pela formação de telurόxido (Figura 2). Outros autores demonstraram o potencial antioxidante de diversos compostos orgânicos de telúrio em vários sistemas *in vitro*, bem como *ex vivo*, utilizando diferentes técnicas e obtendo resultados em baixas concentrações (Engman *et al.*, 1995; Wieslander *et al.*, 1998; Jacob *et al.*, 2000; Tiano *et al.*, 2000; Kanski *et al.*, 2001; Ren *et al.*, 2001; Savegnago *et al.*, 2006).

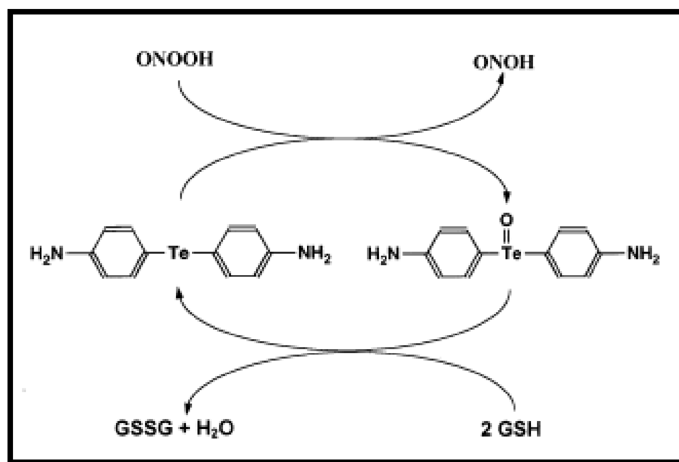


Figura 2: Mecanismo catalítico proposto para a atividade neutralizadora de peroxinitrito dos compostos orgânicos de telúrio (Briviba et al., 1998).

Esta atividade antioxidante dos compostos de telúrio orgânico é muito interessante, especialmente porque quando comparados com análogos contendo selênio, seus efeitos são muito mais pronunciados. Além disso, cada vez mais se comprova a forte relação entre a formação de espécies reativas de oxigênio/nitrogênio e diversas doenças, tornando-se importante a pesquisa de novos e potentes agentes antioxidantes.

II.1.2. Dietil-2-fenil-2-telurofenil vinilfosfonato

O dietil-2 fenil-2-telurofenil vinilfosfonato (DPTVP) (Figura 3) é um composto de telúrio orgânico de estrutura bastante distinta de outros já descritos na literatura. É um β-organocalcogenil vinilfosfonato de grande potencial sintético, visto que combina a conhecida reatividade química dos fosfonatos vinílicos e a capacidade dos calcogenetos vinílicos de facilmente se transformarem em outros compostos orgânicos com retenção

da configuração (Minami e Motoyoshiya, 1992; Comasseto *et al.*, 1997; Zeni *et al.*, 2006).

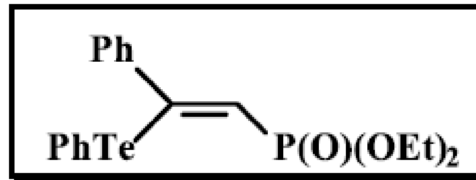


Figura 3: Estrutura química do dietil-2-fenil-2-telurofenil vinilfosfonato (Braga e cols, 2003).

O DPTVP tem sido descrito como um composto com baixa toxicidade em roedores. Em camundongos, administrações diárias por até 12 dias de 250µmol/Kg de DPTVP pela via subcutânea ocasionaram um índice de apenas 20% de mortalidade (De Avila *et al.*, 2006). Nos animais sobreviventes, não foram observadas alterações na atividade da enzima δ- ALA D em fígado, rins e cérebro (De Avila *et al.*, 2006). Além disso, administrações diárias por doze dias de até 500µmol/Kg pela via intraperitoneal em camundongos não causaram mortalidade nos animais nem alterações em diversos parâmetros analisados, tais como níveis de TBARS, atividade de enzimas antioxidantes como SOD e catalase, atividades séricas da AST e ALT e da δ- ALA D (Avila *et al.*, 2007).

In vitro, este telureto vinílico mostrou-se capaz de reagir com grupos sulfidrílicos, aumentando a oxidação do DTT e inibindo a δ- ALA D (De Avila *et al.*, 2006), porém em concentrações bastante elevadas quando comparadas às concentrações utilizadas de ditelureto de difenila, um composto de telúrio extremamente tóxico (Nogueira, Zeni *et al.*, 2004). Aliado a isso, os autores reportaram a potente atividade antioxidante deste composto em fígado, rim e cérebro, uma vez que o DPTVP reduziu a peroxidação lipídica induzida por Fe (II) (De Avila *et al.*, 2006).

II.2. Espécies reativas de oxigênio e nitrogênio

As espécies reativas ou radicais livres são moléculas que possuem um elétron desemparelhado, tornando-se espécies eletrofílicas altamente reativas, que podem reagir com componentes celulares importantes, como proteínas, DNA e lipídeos (Josephy, 1997; Timbrell, 2000).

Essas espécies são produzidas constantemente e sob condições normais nas células eucarióticas, desempenhando funções importantes para o organismo como na coordenação da inflamação e como segundo-mensageiros, por exemplo, (Droge, 2002) (Figura 4). Entretanto, esses processos, ao se exacerbarem em decorrência a alguma doença, acabam causando a excessiva produção das espécies reativas (Halliwell e Gutterridge, 2003).

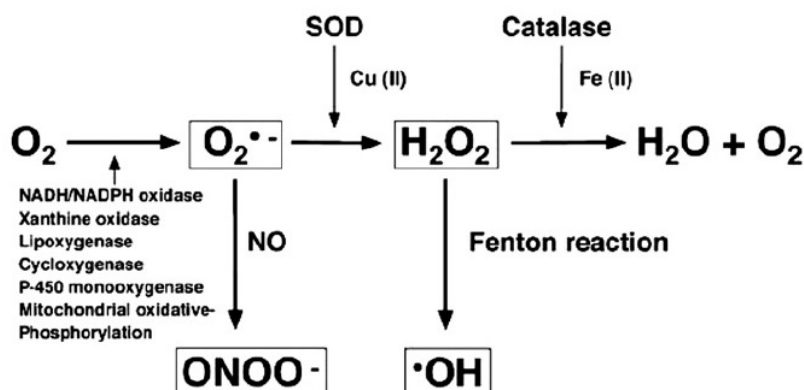
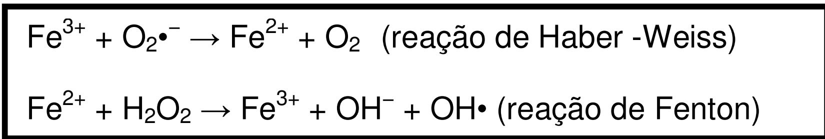


Figura 4: Esquema geral de formação de ERO/ERN nas células eucarióticas.

O superóxido ($O_2\bullet^-$) é gerado pela redução do oxigênio (O_2 – elétron= $O_2\bullet^-$). Este radical é formado por reações de autooxidação na cadeia transportadora de elétrons mitocondrial (Mandelker, 2008). Não é reativa, a menos que entre em contato com outros radicais. Por exemplo, ao entrar em contato com o ON•, reage, ocasionando a formação de peroxinitrito, uma espécie reativa de nitrogênio extremamente reativa (Halliwell *et al.*, 1999), que será descrita logo mais. Além disso, o $O_2\bullet^-$ é capaz de inativar enzimas que possuem um centro ferro-enxofre, causando a liberação do íon ferro. Ferro

e $O_2\bullet^-$ podem reagir via reação de Haber-Weiss, uma reação em cadeia que inclui a reação de Fenton, gerando o radical hidroxil (OH•) (Haber e Weiss, 1932) (Esquema 1).

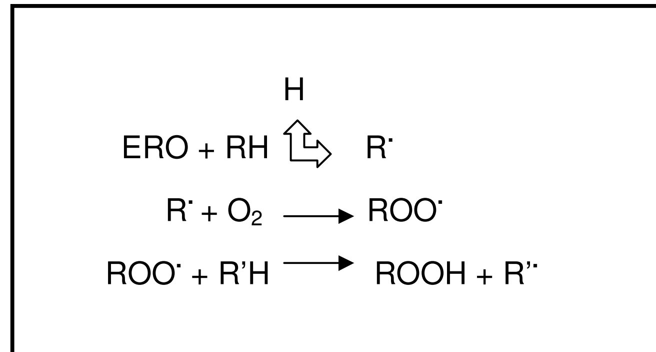


Esquema 1: Reação de Haber-Weiss.

O radical hidroxil é uma espécie altamente reativa. É produzido in vivo por diversos processos e, uma vez formado, reage com qualquer biomolécula presente no seu local de formação (Halliwell e Gutterridge, 2003). O seu ataque ao DNA, por exemplo, produz uma variedade de produtos, incluindo base purínicas e pirimídicas modificadas (Halliwell e Aruoma, 1991).

O peróxido de hidrogênio (H_2O_2) é uma espécie que pode ser gerada pela dismutação do superóxido e por ação de algumas enzimas, como a xantina oxidase (Halliwell e Gutterridge, 2003). Embora o H_2O_2 não seja considerado um radical por não possuir um elétron desemparelhado, é incluído como uma ERO pelas reações que pode desencadear e os radicais que pode gerar. O H_2O_2 pode degradar algumas hemeproteínas (mioglobina e hemoglobina, por exemplo), liberando ferro. A reação do H_2O_2 com metais é amplamente conhecida como a reação de Fenton, como anteriormente demonstrada (Halliwell e Gutterridge, 2003) (esquema 1).

Ainda entre as ERO, há os radicais alcoxil/peroxil, radicais orgânicos formados por reações de peroxidação lipídica (esquema 2). Uma ERO reage com um ácido graxo insaturado (RH), gerando um radical instável (R•). Esse radical reage rapidamente com o oxigênio molecular, gerando radicais livres de ácidos graxos (ROO•). Os novos radicais são também instáveis e podem reagir com outros ácidos graxos (R'H), produzindo mais radicais e até mesmo H_2O_2. Essa reação em cadeia apenas pára quando são formadas espécies estáveis e não reativas (Aikens e Dix, 1991).



Esquema 2: Reações da cascata de peroxidação lipídica.

Dentre as espécies de nitrogênio, as mais danosas são o óxido nítrico (ON•) e o peroxinitrito ($ONOO^-$). O ON• é um gás muito reativo, formado no organismo pela ação da enzima óxido nítrico sintase (NOS), sendo uma importante molécula sinalizadora e presente em diversas partes do corpo (Halliwell *et al.*, 1999). Por ser um gás o ON• se difunde facilmente nas membranas celulares, sendo perigoso em excesso, pois ao reagir com o radical ânion superóxido, gera o $ONOO^-$. O $ONOO^-$ é um agente oxidante e nitrante. Suas propriedades oxidantes atuam em diversas biomoléculas, incluindo proteínas e o DNA (Halliwell *et al.*, 1999). É citotóxico no pH 7,4 e sua adição às células causa morte celular geralmente por apoptose (Halliwell *et al.*, 1999).

Os estudos sobre a toxicidade das ERO/ERN têm sido acompanhados por pesquisas sobre o uso de antioxidantes, de moléculas com atividade neutralizante de espécies reativas e até mesmo de moléculas que estimulem antioxidantes endógenos. Esses estudos são importantes principalmente para o tratamento de doenças nas quais o estresse oxidativo está envolvido, sendo o motivo pelo qual tais estudos hoje estão em evidência na comunidade científica (Halliwell, 2001; Brambilla *et al.*, 2008; Dodd *et al.*, 2008).

II.3. Glutamato

O glutamato é o mais abundante neurotransmissor excitatório no sistema nervoso central. Esse aminoácido é encontrado em altas concentrações (5-15mmol/Kg de tecido, dependendo da área do SNC), estando envolvido em vários processos fisiológicos, tais como aprendizado, memória e a formação de redes neurais durante o desenvolvimento (Fonnum, 1984).

O glutamato é sintetizado nos terminais pré-sinápticos, predominatemente a partir de glutamina por ação da enzima glutaminase, mas também via glutamato desidrogenase e α-cetoglutarato aminotransferase (Kvamme, 1998). É transportado e armazenado em vesículas e subsequentemente liberado por exocitose na fenda sináptica, uma vez que haja despolarização da membrana pré-sináptica, através dos transportadores de glutamato vesicular (VGLUT) (Takamori, 2006). Ao ser liberado, o glutamato exerce usas funçoes fisiológicas ao se ligar aos seus receptores pré e pós-sinápticos (Meldrum *et al.*, 1999). Os receptores são divididos em dois grupos: os metabotrópicos (mGluRs), que são receptores associados a sistemas de segundos-mensageiros intracelulares, principalmente acoplados a proteínas G (Danbolt, 2001); e receptores ionotrópicos, canais iônicos que permeiam cátions através da membrana neural, desencandadeando uma resposta excitatória. Estes receptores são subdivididos em N-metil-D-aspartato (NMDA), ácido α-amino-3-hidróxi-5-metil-4-isoxazol-propiônico (AMPA) e ácido caínico (KA), com base na sua sensibilidade a agonistas específicos (Danbolt, 2001).

A ação do glutamato é finalizada através de sua captação pelos astrócitos ou pelos neurônios pré-sinápticos (Danbolt, 2001). Há basicamente dois tipos de transportadores, os de alta afinidade e os de baixa afinidade. Os transportadores de alta afinidade ao glutamato são Na^+- dependentes e chamados de transportadores de aminoácidos excitatórios (EAAT). Estes são principalmente encontrados nos astrócitos, resposáveis por captar aproximadamente 80% do glutamato extracelular (Robinson e

Dowd, 1997). Já os transportadores de baixa afinidade são Na^+ - independentes, encontradas nas membranas das vesículas sinápticas (Takagaki, 1976).

Os processos de liberação, ligação e captação de glutamato são estritamente orquestados, uma vez que alterações na homeostase do glutamato, especialmente quando este se encontra em altas concentrações na fenda sináptica, podem estar envolvidas em diversas desordens neurológicas, como doença de Huntigton, Mal de Alzheimer, isquemia e epilepsia (Krieglstein, 1997). Nesses processos, o excesso de glutamato superestimula seus receptores, em especial o NMDA, que possui alta permeabilidade ao Ca^{2+} (Danbolt, 2001). O influxo aumentado de Ca^{2+} leva à ativação de proteases, lipases e endonucleases, que acabam causando danos à membrana das mitocôndrias e, consequentemente, reduzem a produção de energia, causam disfunção mitocondrial, estresse oxidativo e morte celular (Akaike *et al.*, 1999). Além disso, a estimulação via NMDA ativa a óxido nítrico sintase e a liberação de NO•, que pode, por sua vez, reagir com o $O_2•^-$ e gerar $ONOO^-$ (Beckman *et al.*, 1990).

Algumas substâncias químicas são capazes de alterar os processos que controlam a homeostase do glutamato, sendo excitotóxicos. Outas substâncias são capazes de mimetizar os efeitos causados pelo glutamato. Um exemplo é o ácido quinolínico (QA), um metabólito endógeno do metabolismo da quinurenina. A neurotoxicidade do QA é mediada pela ativação de receptores NMDA, causando influxo de Ca^{2+} e a mesma subseqüente cascata de efeitos causada pelo excesso de glutamato (Stone, 1991). Outro exemplo é o nitroprussiato de sódio (SNP), um complexo coordenado de íons Fe^{2+}, cinco ânions cianetos e um cátion NO^+. O SNP ($[NO–Fe–(CN)_5]$) é uma substância química que, em meio aquoso intracelular, libera NO• à longo prazo (Bates *et al.*, 1991), assim como ocorre durante a estimulação de receptores NMDA. Além disso, o SNP também gera o ânion ferricianeto ($[(CN)_5–Fe]^{-3}$), que pode reagir com o H_2O_2 e, via reação de Fenton, gerar radicais $OH^·$ (Graf *et al.*, 1984).

II.4. Manganês

O Manganês é um elemento essencial presente em todos os organismos vivos e é naturalmente encontrada em rochas, solo, água e em alimentos, tais como grãos

integrais, leguminosas, nozes e chás (Maban e Escott-Stump, 1998). É extremamente importante para o funcionamento de algumas enzimas, para o crescimento normal, homeostase e desenvolvimento celular, entre outras funções. Entretanto, seu amplo uso industrial e, mais recentemente, seu uso como um aditivo à gasolina como metilciclopentadienil tricarbonil de Mn (Gerber *et al.*, 2002) tornou-o uma importante fonte de poluição ambiental, o qual vem sendo estudado por diversos pesquisadores quanto aos efeitos que a exposição em longo prazo poderá causar aos humanos (Pfeifer *et al.*, 2004).

II.4.1. Mn e sua essencialidade

O Mn é requerido para o metabolismo normal de aminoácidos, lipídeos, proteínas e carboidratos. Há muitos processos celulares que são Mn-dependentes, como os realizados por enzimas das famílias das oxidorredutases, transferases, hidrolases, liases, isomerases e ligases. Arginase, glutamina sintetase, fosfoenolpiruvato descarboxilase e a Mn-superóxido dismutase são comumente referidas como metaloenzimas. O Mn é essencial para a regulação da glicemia para energia celular, função imune normal, reprodução, digestão, crescimento ósseo e defesa contra o estresse oxidativo, inerente à enzima Mn-SOD. Em conjunto à vitamina K, o Mn auxilia nos processos de cicatrização (Aschner e Dorman, 2006).

O Mn é requerido como cofator para diversas enzimas fundamentais à função do SNC. Para algumas enzimas, outro metal como magnésio e cobre podem funcionalmente substituir o Mn, entretanto para outras o Mn é fundamental para a atividade enzimática normal. Essas enzimas-dependente de Mn incluem diversas proteínas requeridas para a funcionabilidade apropriada de neurônios e da glia, como a glutamina sintetase, piruvato descarboxilase, superóxido dismutase (SOD), arginase e serina/proteína fosfatase (Christianson, 1997; Takeda, 2003).

Não há uma dose diária recomendada (DDR) de Mn. Entretanto, foi estabelecida uma ingestão diária recomendada e segura de 2-5mg/dia para adultos (Greger, 1998). Para adultos homens, a dose adequada é de 2,4mg/dia, enquanto que para mulheres foi definida a dose de 1,8mg/dia. Essas doses foram definidas após

algumas observações sobre a absorção de Mn no trato gastrointetsinal baixo em homens x mulheres (Finley *et al.*, 1994). Acredita-se que a lactação e a gestação causam aumento na requisição de Mn. Além disso, de acordo com os diferentes estágios iniciais de vida, diferentes doses de Mn são indicadas: para recém-nascidos (menos de seis meses de idade), a ingestão adequada de Mn é de 3µg/dia; dos 7 aos 12 meses, 600µg/dia. Entre 1-3 e 4-8 anos de idade, as doses diárias de Mn indicadas são aproximadamente 1,2 e 1,5 mg/dia, respectivamente (Finley *et al.*, 1994).

Considerada a essencialidade do Mn, a ingestão inadequada do metal pode resultar em uma série de problemas, desde prejuízo geral do crescimento até defeitos na formação muscular e esquelética (Aschner *et al.*, 2007). Redução de fertilidade e defeitos no nascimento, bem como tolerância anormal à glicose e metabolismo lipídico e de carboidratos alterado têm sido associados com restrição severa à ingestão de Mn (Keen *et al.*, 1999).

II.4.2. Mn e neurotoxicidade

John Couper foi o primeiro a reportar os efeitos neurológicos associados com a exposição ao Mn em 1837, quando descreveu fraqueza muscular, tremor límbico, fala em sussurro, salivação, postura não-ereta em cinco trabalhadores em uma plantação na França (Couper, 1837b). Ele denominou essa coleção de sintomas de “doenças dos trituradores de Mn”, a qual foi chamada de manganismo mais tardiamente. Historicamente, a neurotoxicidade por Mn tem sido comumente associada com atividades como a mineração, o manufaturamento de baterias e a produção de aço (Aschner *et al.*, 2007). Em humanos, foi postulado que há um espectro de efeitos neurocomportamentais e neurofisiológicos associados com a toxicidade de Mn, incluindo ambos sintomas clínicos e subclínicos. Além disso, alguns estudos reportaram efeitos adversos hematológicos, endócrinos ou no sistema reprodutor masculino após exposição ao Mn (Aschner e Dorman, 2006).

O manganismo é uma síndrome neurológica que se assemelha à doença de Parkinson. As similaridades entre as manifestações da doença de Parkinson e o manganismo incluem a presença de bradiscinesia generalizada, rigidez, e

dissimilaridades tais como tremor freqüente, distonia, uma propensão particular de cair para trás, falência na resposta ao tratamento com levodopa e uma característica específica de caminhar sobre os dedos dos pés e com os cotovelos dobrados no manganismo (Calne *et al.*, 1994). As semelhanças entre as duas desordens podem ser explicadas pelo fato de que os gânglios basais acumulam a maior parte do Mn em excesso em comparação a outras regiões do cérebro (Dobson *et al.*, 2004). Já as diferenças se dão pelas vias que são atingidas diretamente: na DP, há perda de neurônios dopaminérgicos dentro da substância negra (Aschner *et al.*, 2007). Já no manganismo, há preservação da via dopaminérgica nigroestriatal, porém os danos ocorrem abaixo dessa via (Calne *et al.*, 1994).

As áreas cerebrais que mais acumulam Mn são coincidentemente as que mais acumulam ferro também. Au et al. (2008) descreveram que isso se deve a uma concentração mais elevada de transportadores de metais divalentes do tipo 1, resposáveis pela entrada de Mn e Fe no sistema nervoso central (Au *et al.*, 2008). As áreas que mais acumulam Mn são os gânglios basais e o globus pallidus, ricas em neurônios dopaminérgicos, responsáveis pelo controle motor (Aschner *et al.*, 2007). O dano causado pelo Mn nessas áreas justifica os efeitos extrapiramidais e a disfunção motora que primatas humanos e não-humanos e roedores apresentam após serem expostos ao metal (Newland, 1999).

O mecanismo pelo qual o Mn causa seus efeitos neurotóxicos ainda não é completamente compreendido. A primeira e mais difundida teoria é pela oxidação da dopamina (DA) em neurônios dopaminérgicos pelo Mn (Figura 5). O Mn é um potente agente oxidante, acelerando a oxidação da DA a o-quinona, que instantaneamente se cicliza e forma o aminocromo (Archibald e Tyree, 1987). Essa reação é irreversível e explica a diminuição drástica de DA observado em modelos animais (Segura-Aguilar e Lind, 1989). O aminocromo sofre uma redução de um elétron via NADPH, gerando o radical leucoaminocromo o- semiquinona, uma reação reversível que gera radical ânion superóxido (Segura-Aguilar *et al.*, 2001). Além disso, o leucoaminocromo o-semiquinona é um metabólito muito reativo, capaz de se auto-oxidar na presença de

oxigênio e metais de transição como Mn e Fe, iniciando o processo cíclico (Baez *et al.*, 1995).

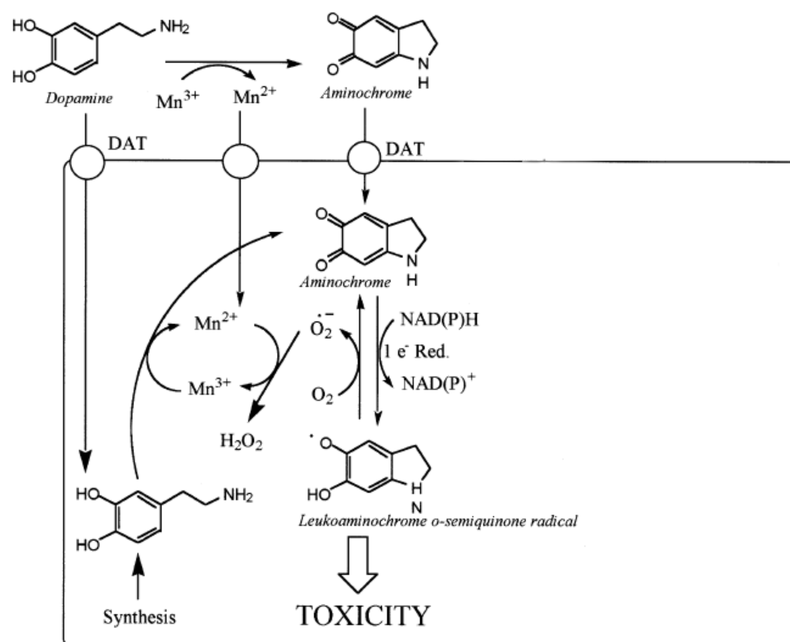


Figura 5: Mecanismo de oxidação da dopamina pelo Mn.

Intracelularmente, a organela que acumula Mn é a mitocôndria. A entrada do metal se dá através de transportadores de Ca^{2+}. Este acúmulo afeta a função mitocondrial, por basicamente três razões: (1) inibição da transmissão de energia; (2) pela indução de mutações no DNA mitocondrial e (3) por aumento das ERO. O Mn inibe a fosforilação oxidativa pela inibição dos complexos I ao IV da cadeia transportadora de elétrons, causando a diminuição da produção de energia (Zhang *et al.*, 2004). A inibição da cadeia transportadora, por sua vez, eleva a taxa de produção de ERO, como o ânion superóxido e radical hidroxil (Boveris e Chance, 1973). Além disso, o Mn causa alterações na homeostase do Ca^{2+}, uma vez que utiliza o mesmo transportador, do tipo uniporter, para entrar na mitocôndria, causando aumento deste dentro da organela. Esse aumento de Ca^{2+} pode levar à abertura do poro de transição de permeabilidade mitocondrial, com conseqüente inchaço da organela, ruptura da membrana externa e

liberação de inúmeros fatores apoptogênicos para o citosol, causando morte celular (Green e Reed, 1998; Gavin *et al.*, 1999)

Entretanto, alguns estudos sugerem que o sistema glutamatérgico seja primariamente envolvido, uma vez que o acúmulo de Mn no cérebro produz um tipo de neuropatologia consistente com um mecanismo excitotóxico (Brouillet *et al.*, 1993). Os astrócitos desempenham um papel importante nesse mecanismo, uma vez que são responsáveis por captar a maior parte do glutamato liberado durante a sinapse (cerca de 80%) (Rutledge *et al.*, 1998). Diferente dos neurônios, os astrócitos possuem a habilidade de captar e acumular Mn em altas concentrações por possuírem um sistema transportador específico e de alta afinidade (Aschner *et al.*, 1992). Dentro dos astrócitos, o Mn causa disfunção mitocondrial e ativação de cascata apoptótica (Yin *et al.*, 2008), levando ao desenvolvimento de astrocitose, condição na qual os astróctos apresentam alterações metabólicas e morfológicas, levando ao decaimento das funções realizadas por essas células gliais (Pentschew *et al.*, 1963). Dessa maneira, a captação de glutamato fica prejudicada, levando ao aumento da concentração sináptica desse neurotransmissor (Hazell e Norenberg, 1997). Além disso, Erikson e Aschner (2002) demonstraram que a diminuição da captação de glutamato pelo Mn também involve a diminuição da expressão do RNA mensageiro do principal transportador de glutamato, o transportador de glutamato/aspartato (GLAST), o que também leva a concentrações elevadas de glutamato e seus efeitos excitotóxicos (Erikson e Aschner, 2002)

II.5. Acetaminofeno (APAP)

O Paracetamol ou acetaminofeno é certamente um dos anagésicos/antipiréticos mais comumente utilizado na medicina. Sua ascensão se deve à descoberta do desenvolvimento de síndrome de Reye associado ao uso de aspirina (Committee on Infectious Diseases, 1982). Entretanto, o uso aumentado e desenfreado desse medicamento vem sendo associado a inúmeros casos de tentativas de suicídio e de transplantes de fígado por cirrose hepática causada por intoxicação aguda e crônica com APAP (Larson *et al.*, 2005). Tais efeitos vêm sendo investigados por diversos grupos de pesquisa e apontam as causas para o estresse oxidativo que o APAP pode

causar, bem como sugerem tratamento eficiente com antioxidantes, como a N-acetilcisteína (NAC) (Linden e Rumack, 1984).

II.5.1. Mecanismo analgésico/antipirético do APAP

Apesar de sua popularidade, o mecanismo pelo qual o acetoaminofeno atinge seus efeitos sobre a febre e a dor ainda não é completamente compreendido. O mecanismo mais conhecido é pela inibição da via das ciclooxigenases (Figura 6). Nessa via, prostaglandinas (PG), substâncias pró-inflamatórias, são produzidas. As PGs são mediadores químicos responsáveis por transmitir a dor e por sinalizar ao cérebro, especialmente no hipotálamo, que um processo inflamatório está ocorrendo no corpo, resultando no aumento da temperatura corpórea (pirexia) (Kluger, 1991). Ao inibir essa rota, o APAP e outros antiinflamatórios não – esteroidais (AINES) bloqueiam a produção de PGs e, conseqüentemente, a febre e a dor.

Mecanismos alternativos para a ação do APAP têm sido propostos. Tjolsen et al. (1991) demonstraram o envolvimento de receptores serotoninérgicos subtipo $5HT_3$ na resposta antinociceptiva do APAP, interferindo com vias serotoninérgicas descendentes da dor (Tjolsen *et al.*, 1991). Além disso, o APAP também pode inibir a síntese de óxido nítrico (NO) indiretamente. Bjorkman (1995) demonstrou que o APAP interfere com a nocicepção associada à ativação espinhal de receptor NMDA, dessa maneira inibindo a síntese e a ação espinhal do NO (Bjorkman, 1995).

II.5.2. Hepatotoxicidade causada por APAP

O APAP é efetivo e seguro quando consumido conforme o recomendado (1-4g por dia). Em 1966, entretanto, houve os primeiros relatos de necrose fatal e não fatal seguida por excessiva ingestão de altas doses do analgésico (Davidson e Eastham, 1966; Thomson e Prescott, 1966). Hoje está bem documentada que a ingestão de uma única dose maior do que 10g pode causar dano hepático severo ou até mesmo fatal (Mcjunkin *et al.*, 1976). Tem sido também sugerido que a hepatotoxicidade por APAP

pode ocorrer com o uso de doses terapêuticas sob algumas condições, como uso de álcool e sob o estado de jejum (Zimmerman, 1981).

A hepatotoxicidade por APAP ocorre devido a sua metabolização (Figura 7). Em doses normais, 2% do APAP é excretado diretamente na urina. O remanescente é metabolizado hepaticamente por dois mecanismos: 90% é metabolizado por conjugação – dois terços por glicuronidação (UDP-glicuronosiltransferases) e um terço por sulfatação (sulfotransferases) (Forrest *et al.*, 1982; Manyike *et al.*, 2000). Os conjugados inativos são excretados na urina e na bile. Aproximadamente 5 a 9% do APAP sofre metabolização por reações de fase 1 Grupos polares são adicionados por oxidação, redução ou hidrólise. No caso do APAP, ocorre uma conversão oxidativa por meio dos citocromos CYP1A2, CYP2A6, CYP2E1 e CYP3A4, gerando o metabólito tóxico N-acetil-p- benzoquinonaimina (NAPQI) (Chen *et al.*, 1998). Pouco NAPQI é produzido quando doses terapêuticas são utilizadas, entretanto doses excessivas de APAP levam à saturação do processo de metabolização pelas vias do ácido glicurônico e pela sulfatação, sobrecarregando o sistema do citocromo e aumentando a produção desse metabólito tóxico (Makin e Williams, 1994).

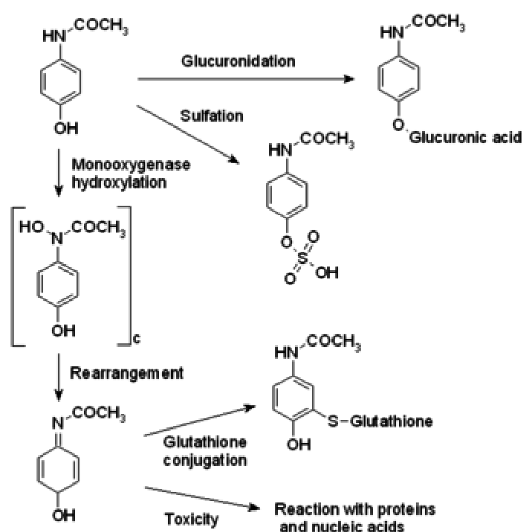


Figura 7: metabolização do APAP e seu mecanismo de hepatotoxicidade e morte celular.

O NAPQI é uma espécie de dois elétrons altamente reativa que pode agir como um eletrófilo ou um oxidante, sendo rapidamente conjugado com a glutationa (GSH) intracelular, a fim de ser eliminado de forma atóxica pelos rins (Mitchell, Jollow, Potter, Gillette *et al.*, 1973). Entretanto, as reservas de GSH são limitadas, sendo depletadas na tentativa de detoxificar o NAPQI produzido em excesso (Mitchell, Jollow, Potter, Gillette *et al.*, 1973). Quando as reservas de GSH estão reduzidas de 70 a 80%, a capacidade de detoxificação do fígado fica excedida e o NAPQI se acumula, interagindo com e destruindo os hepatócitos e outras células (Prescott, 1983). Na ausência de GSH, o NAPQI começa a se ligar em resíduos de cisteína de macromoléculas dos hepatócitos, gerando adutos (Pumford *et al.*, 1989), os quais são o processo inicial e irreversível no desenvolvimento do dano hepático (Gibson *et al.*, 1996).

A depleção de GSH contribui para o estresse oxidativo celular (Jaeschke *et al.*, 2003). Quando o NAPQI liga-se a alvos celulares críticos, como as proteínas mitocondriais, ocorre disfunção mitocondrial e perda de ATP celular (Harman *et al.*, 1991). Os hepatócitos sofrem subseqüentemente falência energética, alteração na homeostase do cálcio, depleção de ATP, dano ao DNA e modificação de proteínas intracelulares (Andersson *et al.*, 1990; Jaeschke e Bajt, 2006). Estes eventos levam à morte celular por necrose.

Justificativa

Tendo em vista a baixa toxicidade do DPTVP, seu potencial antioxidante in vitro e a falta de estudos na literatura sobre possíveis propriedades farmacológicas do composto em modelos animais nos quais o estresse oxidativo esteja envolvido, se torna necessário investigar os as ações do composto em modelos de neuro e hepatotoxicidade, bem como investigar o possível mecanismo de ação pelo qual o composto age como antioxidante nesses modelos.

III. Objetivos

III.1. Objetivo Geral

O Objetivo da presente tese é avaliar as possíveis atividades neuro e hepatoprotetora do dietil-2-fenil-2-telurofenil vinilfosfonato, bem como determinar o potencial antioxidante deste composto através da avaliação da atividade neutralizadora ou "scavenger" de ERO e ERN em roedores.

III.2. Objetivos específicos:

Os objetivos específicos deste trabalho são:

1) Avaliar, *in vitro*, a atividade antioxidante do dietil-2-fenil-2-telurofenil vinilfosfonato frente a pró-oxidantes que afetam o sistema glutamatérgico tais como o nitroprussiato de sódio (SNP) e o ácido quinolínico (QA);
2) Determinar, *in vitro*, se o dietil-2-fenil-2-telurofenil vinilfosfonato compromete o sistema glutamatérgico através de ensaios como captação, liberação e união específica de [^{3}H] glutamato;
3) Determinar, in vivo, os efeitos da administração crônica de $MnCl_2$ na água de beber de ratos sobre a atividade locomotora e exploratória desses animais, bem como se o co-tratamento com DPTVP pode reverter essas alterações;
4) Investigar, ex vivo, os efeitos do tratamento com DPTVP contra a neurotoxicidade induzida por Mn através de parâmetros bioquímicos tais como peroxidação lipídica, viabilidade mitocondrial, níveis de ERO, captação de [^{3}H] glutamato, atividade da SOD e níveis de Mn em córtex, hipocampo e estriado;

5) Averiguar, *ex vivo*, a possível atividade hepatoprotetora do DPTVP contra os parâmetros bioquímicos e histopatológicos alterados pelo APAP nas doses de 200 e 300mg/Kg;

6) Avaliar, *in vitro*, se o DPTVP possui atividade neutralizadora ou “scavenger” contra ERO e ERN e sugerir um possível mecanismo de ação.

IV. 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 nos próprios artigos. O artigo 1 está na forma como foi publicado na revista, enquanto os manuscritos 1, 2 e 3 estão na forma como foram submetidos às revistas.

IV.1. UM COMPOSTO ORGÂNICO DE TELÚRIO COM ATIVIDADE ANTIOXIDANTE CONTRA AGENTES EXCITOTÓXICOS SEM EFEITOS NEUROTÓXICOS NO CÉREBRO DE RATOS

Artigo 1

AN ORGANOTELLURIUM COMPOUND WITH ANTIOXIDANT ACTIVITY AGAINST EXCITOTOXIC AGENTS WITHOUT NEUROTOXIC EFFECTS IN BRAIN OF RATS

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Research report

An organotellurium compound with antioxidant activity against excitotoxic agents without neurotoxic effects in brain of rats

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Abstract

The glutamatergic system is an important target in many neurodegenerative diseases and for several neurotoxic drugs. Organotellurium compounds are often very good free radical scavengers' agents. Recently, we reported that diethyl-2-phenyl-2-tellurophenyl vinylphosphonate is a compound with low toxicity *in vitro* and *in vivo*, as well as also possesses antioxidant activity against iron-induced lipid peroxidation. The aim of this study was to evaluate *in vitro* the antioxidant and mitochondrial protective effect of this organotellurium compound against quinolinic acid (QA) and sodium nitroprusside (SNP), and to evaluate the *in vitro* actions of this organotellurium compound in the glutamatergic system in brain of rats. We observed that the telluro vinylphosphonate possess an antioxidant activity against QA and SNP at micromolar concentrations. When tested at antioxidant concentrations (from 2 to 10 μM), the compound does not affect the mitochondrial viability and [^{3}H]glutamate uptake in slices from cerebral cortex, hippocampus and striatum, [^{3}H]glutamate release from synaptosomal preparations and [^{3}H]glutamate binding in membrane preparation. Our data suggest that the telluro vinylphosphonate act as an antioxidant in the central nervous system *in vitro* with no effects on the glutamatergic system; nevertheless more studies in different models of brain injury must be performed in order to corroborate our findings.
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Keywords: Diethyl-2-phenyl-2-tellurophenyl vinylphosphonate; Antioxidant; Glutamate; Tellurium; Neuroprotection

1. Introduction

Because of its widely distribution in the central nervous system, glutamate is considered the most important excitatory neurotransmitter in mammals. Glutamate is involved in many brain functions as development, aging, learning and physiological integration among brain structures [44,48,54]. Therefore, excessive concentrations of glutamate leads to a cascade of events namely excitotoxicity. In order to maintain glutamate homeostasis, the CNS strictly regulates the fine balance between glutamate release and glutamate uptake. Glutamate is synthesized in the neuronal pre-synaptic cytoplasm by the glutaminase and translocated into the vesicle lumen via a glutamate transport protein located on the vesicle [73]. In response to neuronal activity, the vesicle fuses with plasma membrane, releasing their content. In the synaptic cleft, the neurotransmitter mainly acts when binds to the glutamate receptors [7,11]. When glutamate is released in the synaptic cleft, it is uptaked by specific high-affinity Na^+-dependent amino acid transporters (EAAT), which are mainly present in glial cells, and metabolized by the glutamine pathway, transported as glutamine to the neurons and stored as glutamate now in the vesicles of pre-synaptic neuron to be released again [18,24]. In that way, alterations in the neuronal membrane potential or disturbances in the glutamate uptake, could lead to excessive concentration of glutamate in the synaptic cleft and cause excitotoxicity [4,18].

It is known that neuronal degeneration which is caused by glutamate involves the activation of the glutamatergic receptor NMDA type. The excessive glutamate concentration causes massive NMDA receptor stimulation, which has high permeability to Ca^{+2} [36]. Subsequently high Ca^{+2} influx leads to a cascade of biochemical events, as lipolysis, proteolysis, free radical formation and finally neuronal death [17,78]. In this context, quinolinic acid (QA), an endogenous neuroactive metabolite of the kynurenine pathway, exhibits agonistic properties of the NMDA receptor [67,72], producing a specific

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pattern of neurodegeneration and neurochemical alterations. The toxicity of QA is mediated by sustained activation of the NMDA receptor and Ca^{+2} channels, increasing intracellular Ca^{+2} concentrations, leading to ATP depletion, mitochondrial dysfunction, oxidative stress and cell damage [2]. Some studies demonstrated *in vitro* and *in vivo* prooxidative effects of QA, specially the stimulation of the lipid peroxidation in the brain [61,63]. Otherwise, the lipid peroxidation induced by QA is reduced by antioxidant agents [64,79], as well as by MK-801, an antagonist of NMDA receptor, confirming the involvement of the receptor in the oxidative damage evoked by QA [57,65].

Other proposed mechanism of toxicity induced by Ca^{2+} influx is due to the stimulation of the neuronal nitric oxide synthase (NOS), which is a Ca^{+2}/calmodulin requiring enzyme, generating NO radical ($NO^{\bullet}$). This radical can easily produces, together with superoxide anion radical ($O_2^{\bullet -}$), peroxynitrite ($ONOO^-$), thus leading to lipid peroxidation and production of additional free radicals [10]. In fact, sodium nitroprusside (SNP) is a good chemical inducer of lipid peroxidation in brain tissue [59], since it release in a short-lasting time $NO^{\bullet}$ in tissue preparations [9,39].

Organotellurium compounds have been reported as excellent antioxidants in several models of oxidative stress [14,21,31], especially in brain [5,33]. Conversely, organotellurium compounds are described as neurotoxic [26,37,50], being one of them, diphenyl ditelluride, a very toxic compound to glutamatergic system, which diminishes the potential pharmacological interest in these compounds. Otherwise, we recently reported that a vinylic telluride, the diethyl-2-phenyl-2-tellurophenyl vinylphosphonate, did not present significant toxic effects when administered subcutaneously or intraperitonealy into mice [5,6]. In addition, telluro vinylphosphonate showed a potent antioxidant effect against iron-induced lipid peroxidation *in vitro* [5].

Considering the antioxidant activity depicted by the telluro vinylphosphonate compound, becomes necessary to determine if this organotellurium compound has ability to diminish the oxidative damage in brain, once several brain disorders have been related to ROS production. In this study, we investigated the antioxidant effects of the telluro vinylphosphonate in brain caused by QA and SNP, since both chemicals are able to mimetize the oxidative stress conditions caused by excessive stimulation of NMDA receptor by glutamate. Moreover, assuming that glutamatergic system is a concerning matter, we aimed to examine if the telluro vinylphosphonate alters *per se* the homeostasis of the neurotransmitter in the synaptic cleft, through assays that evaluate the glutamatergic system, as [^{3}H]glutamate uptake, release and binding in brain of rats.

2. Materials and methods

2.1. Chemicals

[^{3}H]Glutamic acid (1 Ci/mL) was purchased from Amersham Biosciences All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Diethyl-2-phenyl-2-tellurophenyl vinylphosphonate (Fig. 1) synthesis was performed by addition of alkynylphosphonates to a solution of sodium organyl tellurolate, prepared by the reduction of diorganyl ditellurides with sodium borohydride in ethanol at room temperature [13].

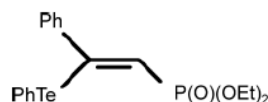

Fig. 1. Chemical structure of diethyl-2-phenyl-2-tellurophenyl vinylphosphonate.

2.2. Animals

Adult Wistar rats from our own breeding colony were maintained in an air conditioned room (20–25 °C) under natural lighting conditions with water and food (Guabi-RS, Brasil) *ad libitum*. All experiments were conducted in accordance with the Guiding Principles for the Use of Animals in Toxicology, adopted by the Society of Toxicology in July 1989.

2.3. QA and SNP-induced lipid peroxidation

Animals were killed by decapitation. Brain was removed, and the whole brain was used or dissected into three specific areas (cortex, hippocampus and striatum) and then homogenized (1:10) in Tris–HCl 10 mM buffer, pH 7.4 and centrifuged at 4000 × *g* for 10 min at 4 °C. The low speed supernatant fraction obtained (S1) was used for TBARS measurements.

The antioxidant effect of the telluro vinylphosphonate was evaluated against SNP (5 μM) and QA (1 mM) through measurements of thiobarbituric acid reactive substances production, according to Ohkawa et al. [53], using vehicle (DMSO), 0.4, 1, 2, 4 and 10 μM of the telluro vinylphosphonate. The homogenates were pre-incubated for 1 h at 37 °C in a buffered medium with the telluro vinylphosphonate in the different concentrations, to evaluate the compound effects on the basal TBARS production or pre-incubated with the compound plus QA or SNP, to observe the effect of the organotellurium compound in the lipid peroxidation induced by both agents. Then, SDS 8.1%, acetic acid/HCl buffer and thiobarbituric acid 0.6% were added to the tubes, and incubated for 1 h at 95 °C. TBARS formation were determined spectrophotometrically at 532 nm, using Malondialdehide as standard.

2.4. Mitochondrial viability

Neuronal injury in cortex, hippocampus and striatum was quantified by measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a dark violet formazan product [47] by mitochondrial dehydrogenases. Slices (0.4 mm) of the brain areas were obtained by transversally cuts of using a McIlwain chopper. Previously, a curve–response of mitochondrial viability to telluro vinylphosphonate, QA and SNP was determined, and then it was chosen the concentration of SNP and QA that caused mitochondrial damage to determine whether telluro vinylphosphonate would be able to prevent this effect. Slices were pre-incubated with telluro vinylphosphonate (1, 5, 10, 50 and 100 μM), or SNP (5, 10, 50 and 100 μM) by 1 h or QA (100, 500 and 1000 μM) by 2 h in oxygenated buffer, containing (in mM): 118 NaCl, 1.2 KH_2PO_4, 4.7 KCl, 2.5 $CaCl_2$, and 1.17 $MgSO_4$. After incubation, the slices were washed twice with 1 mL of buffer. MTT reduction assays were performed in plates containing 500 μL of buffer, and the reaction was started by adding 0.5 mg/mL MTT. After 45 min of incubation at 37 °C, medium was removed and the slices dissolved in dimethylsulfoxide (DMSO). The rate of MTT reduction was measured spectrophotometrically at a test wavelength of 570 nm and a reference wavelength of 630 nm.

2.5. [^{3}H]Glutamate uptake

Slices (0.4 mm) were obtained by transversally cuts of cortex, hippocampus and striatum using a McIlwain chopper. The experiments were made using one slice and in triplicates. The slices were pre-incubated with DMSO, 0, 1, 5, 10,

50 and 100 μM of telluro vinylphosphonate for 15 min, and then washed with a HBSS solution containing (mM): 137 NaCl, 0.63 Na_2HPO_4, 4.17 $NaHCO_3$, 5.36 KCl, 0.44 KH_2PO_4, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$ and 5.55 glucose, adjusted to pH 7.2. The glutamate uptake was performed according to Frizzo et al. [23] with some modifications. Briefly, uptake was carried out at 35 °C by adding 100 μM of unlabeled glutamate and 0.33 μCi/mL [^{3}H]glutamate. The reaction was stopped after 7 (cortex), 5 (hippocampus) and 3 (striatum) minutes by washing two times with 1 mL cold HBSS, immediately followed by addition of 0.5 N NaOH, which was kept overnight. Na independent uptake was determined by using choline chloride instead of NaCl, which was subtracted from the total uptake to obtain the Na^+-dependent uptake. Incorporated radioactivity was determined with a Packard scintilator (TRI CARB 2100 TR). All experiments were performed in triplicate.

2.6. Synaptosomal preparation

Synaptosomal preparations were obtained by isotonic Percoll/sucrose discontinuous gradients at 4 °C, as previously described [20] with few modifications. Briefly, homogenates (10%, w/v) from forebrain were made in 0.32 M sucrose, 1 mM EDTA and 6.25 mM DDT (pH 7.4), and centrifuged at 800 × *g* for 10 min. The supernatant containing synaptosomes were subjected to 23, 15, 7 and 3% Percoll solution density gradient centrifugation at 24,000 × *g* for 10 min. The synaptosomal fractions were isolated, suspended and homogenized in buffered HBSS containing low K^+ (pH 7.4), containing in mM: 133 NaCl, 2.4 KCl, 1.2 KH_2PO_4, 1.09 $MgSO_4$, 27.7 HEPES, 1.2 glucose and 0.001 $CaCl_2$ and centrifuged at 21,000 × *g* for 15 min. The supernatant was removed and the pellet gently resuspended in HBSS buffer. The synaptosomal fraction used contained approximately 2.2 mg of protein/mL. This fraction also contained approximately 5% contamination with fragments of the inner and outer mitochondrial membranes, microsomes, myelin, as well as glial plasmatic membranes [45].

2.7. Synaptosomal [^{3}H]glutamate release

Determination of [^{3}H]glutamate release was accomplished as described by Migues et al. [45]. Prior to the release assay, synaptosomal preparations from rat forebrain were loaded with labeled [^{3}H]glutamate for 15 min at 37 °C. Incubation was performed in a non-depolarizing medium (low potassium), containing, in mM: HEPES 27, NaCl 133, KCl 2.4, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 12, $CaCl_2$ 1.0 in the presence of 0.5 μM of glutamate (0.1 μCi [^{3}H]glutamate). Aliquots of labeled synaptosomal preparations (1.4 mg protein) were centrifuged at 16,000 × *g* for 1 min. Supernatants were discarded, and the pellets were washed four times in the medium by centrifugation at 16,000 × *g* for 1 min (at 4 °C). To assess the basal release of [^{3}H]glutamate, the final pellet was resuspended in the same buffer and incubated for 1 min at 37 °C in the absence (basal or DMSO) and in the presence of telluro vinylphosphonate (1, 5, 10, 50 and 100 μM). K^+-stimulated [^{3}H]glutamate release was assessed as described for basal release, except that the incubation medium contained 40 mM KCl to induce synaptosomal depolarization. Incubation was terminated by immediate centrifugation (16,000 × *g* for 1 min). Radioactivity present in supernatants and pellets was separately determined. [^{3}H]glutamate release was calculated as the percentage of total amount of radiolabel glutamate present at the start of the incubation period in preloaded synaptosomes. The total amount of synaptosomal [^{3}H]glutamate release under these conditions was approximately 9.9 nmol/(mg(protein) min).

2.8. Brain membrane preparation

All binding assays were performed using brain synaptic membranes prepared as described by Jones and Mathus [32] using a sucrose gradient for differential centrifugation, and stored adequately prior to use. On the day of the binding assay, the membranes were thawed in a 30 min water bath (37 °C), homogenized with three volumes of Tris–HCl buffer (5 mM, pH 7.4), and centrifuged three times at 27,000 × *g* for 15 min. The final pellet was resuspended in the same buffer in order to yield a protein concentration of 1–2 mg/mL and was used for the binding assay.

2.9. [^{3}H]Glutamate binding assay

Membranes were incubated in 0.5 mL reaction mixture containing 500 mM Tris–acetate, pH 7.4, 40 nM [^{3}H]glutamate and in the absence (basal or DMSO) and in the presence of telluro vinylphosphonate (1, 5, 10, 50 and 100 μM). Incubation was carried out at 30 °C for 30 min and the reaction was stopped by centrifugation at 27,000 × *g* for 15 min. The pellet and the wall of the tube were quickly and carefully washed with ice-cold Milli-Q water. SDS (0.1%) and scintillation liquid were added to the dry pellet and incorporated radioactivity was determined [70]. In all binding experiments, nonspecific binding was determined by adding 1000 times non-labeled glutamate to the medium in a parallel assay. [^{3}H]glutamate binding control values were 3.6 ± 0.96 pmol/(mg protein per 10 min). Nonspecific binding typically amounted to 20% of total binding. Specific binding was considered to be the difference between total and nonspecific binding.

2.10. Protein determination

Aliquots from the homogenized slices, membranes, homogenates and synaptosomal preparation were separated to protein measurements that were assessed according to Lowry et al. [42].

2.11. Statistical analysis

Statistical significance was assessed by one-way ANOVA, followed by Duncan test for post hoc comparison. Results were considered statistically significant at values of $p < 0.05$.

3. Results

3.1. QA and SNP-induced lipid peroxidation

QA induced a significant increase in TBARS formation in whole brain and in the three brain structures homogenates in approximately 250% from basal levels ($p < 0.001$, Fig. 2A–D). Similarly, SNP induced an increase of approximately 200% ($p < 0.001$, Fig. 3A–D). The telluro vinylphosphonate significantly decreased spontaneous and QA-induced TBARS formation in rat whole brain homogenates ($p < 0.001$, Fig. 2A). In addition, there was a different sensitivity in the brain regions to the antioxidant effect of telluro vinylphosphonate, since in hippocampus the organotellurium compound was able to decrease the QA-induced lipid peroxidation from 1 μM ($p < 0.05$, Fig. 2C), otherwise, in cortex and striatum, the antioxidant action was significant from 2 μM (Fig. 2B and D).

Moreover, the telluro vinylphosphonate significantly decreased spontaneous and SNP-induced lipid peroxidation from 2 μM in the whole brain homogenate and in the cortex ($p < 0.001$, Fig. 3A and B). Nevertheless, in hippocampus and striatum homogenates, the SNP-induced TBARS formation significantly decreased only from 4 μM of the compound ($p < 0.05$, Fig. 3C and D).

3.2. Mitochondrial viability

3.2.1. Curve–responses

Table 1 shows that the telluro vinylphosphonate did not cause mitochondrial damage in slices of the brain regions and at any of the tested concentrations used here. In a plot using different SNP concentrations, we observed that SNP, which induced lipid peroxidation at 5 μM, caused decrease in MTT reduction only from

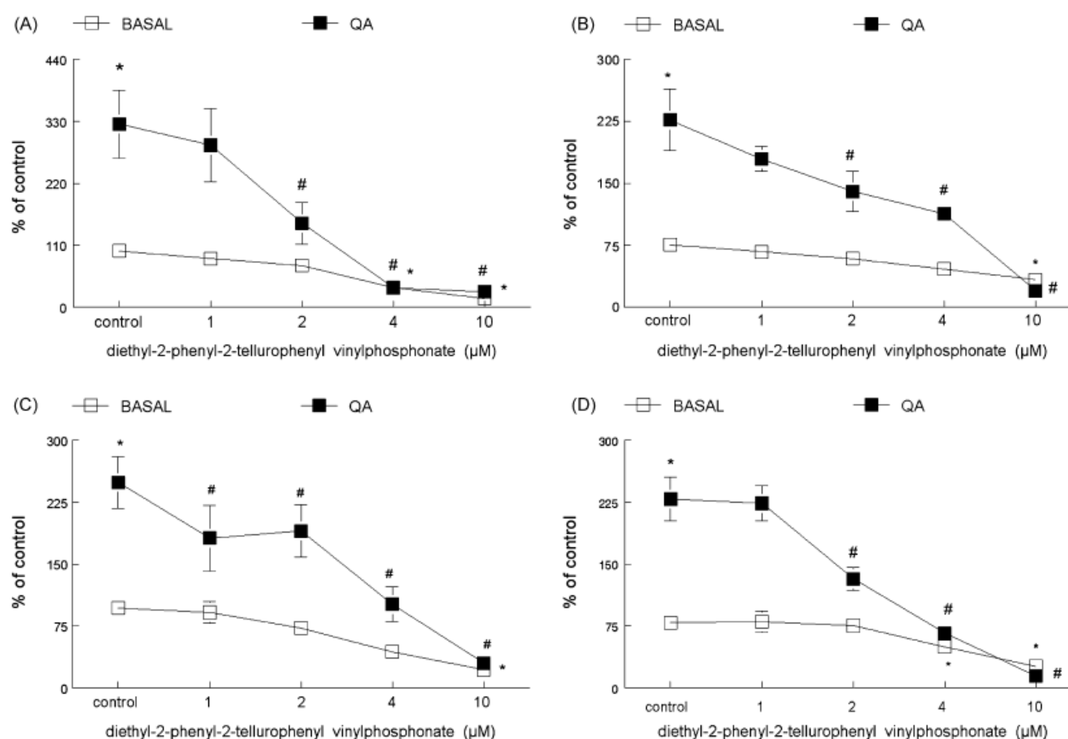

Fig. 2. Effects of diethyl-2-phenyl-2-tellurophenyl vinylphosphonate. On spontaneous (□) and QA-induced (■) TBARS production *in vitro* in whole brain (A), cortex (B), hippocampus (C) and striatum (D) of rats. Results are expressed as percent of control. 100% of control corresponds to 1.46 nmol MDA/g of tissue (A) and 2.0 nmol MDA/g of tissue (B–D). *Indicates statistical difference from the control basal, $p<0.05$; #Indicates statistical difference from control QA-induced TBARS formation, $p<0.05$. All experiments were performed in duplicates ($n=5$).

50 μM ($p<0.05$, data not shown). On the other hand, QA was not able to cause mitochondrial damage at the concentrations used here (Table 2), even when pre-incubated for 2 h.

3.2.2. Protective effect of telluro vinylphosphonate

The organotellurium compound was able to protect cortex slices from the damage induced by 50 μM of SNP from 4 μM ($p<0.05$, Fig. 4A). Meanwhile, in hippocampus and striatum the protective effect was observed only from 40 μM ($p<0.05$, Fig. 4B and C). In striatum, there was a reduction in mitochondrial viability when 0.4 μM of telluro vinylphosphonate was added to the medium ($p<0.05$, Fig. 4C).

Table 1
Curve–response of telluro vinylphosphonate on mitochondrial viability (MTT assay) in cortex, hippocampus and striatum slices

Telluro vinylphosphonate (μM)	MTT (% of control)		
	CTX	HIP	STR
0	100 ± 1.2	100 ± 2.5	100 ± 1.5
0.4	231.45 ± 109.5	174.35 ± 95.3	90.88 ± 38.9
4	112.84 ± 10.19	289.3 ± 81.0	152.88 ± 42.7
40	89.4 ± 2.5	150.44 ± 55.4	99.8 ± 53.1

Data are expressed as mean ± S.E.M for four independent experiments.

3.3. [^{3}H]Glutamate uptake

Na^+-dependent [^{3}H]glutamate uptake was not altered striatum slices (Fig. 5C). However, at 100 μM of the telluro vinylphosphonate, [^{3}H]glutamate uptake was decreased in cortex ($p<0.05$, Fig. 5A) and hippocampus slices ($p<0.05$, Fig. 5B).

3.4. Synaptomal [^{3}H]glutamate release

Fig. 6 shows that at the concentration of 100 μM the organotellurium compound increased both basal and K^+-

Table 2
Curve–response of QA on mitochondrial viability (MTT assay) in cortex, hippocampus and striatum slices

QA (μM)	Mitochondrial viability (% of control)		
	CTX	HIP	STR
0	100 ± 3.2	100 ± 5.6	100 ± 7.3
100	109.73 ± 26.42	108.81 ± 12.45	115.99 ± 20.17
500	134.76 ± 48.44	101.54 ± 17.33	158.08 ± 25.15
1000	125.14 ± 65.11	100.3 ± 60.6	165.91 ± 24.34

Data are expressed as mean ± S.E.M for four independent experiments.

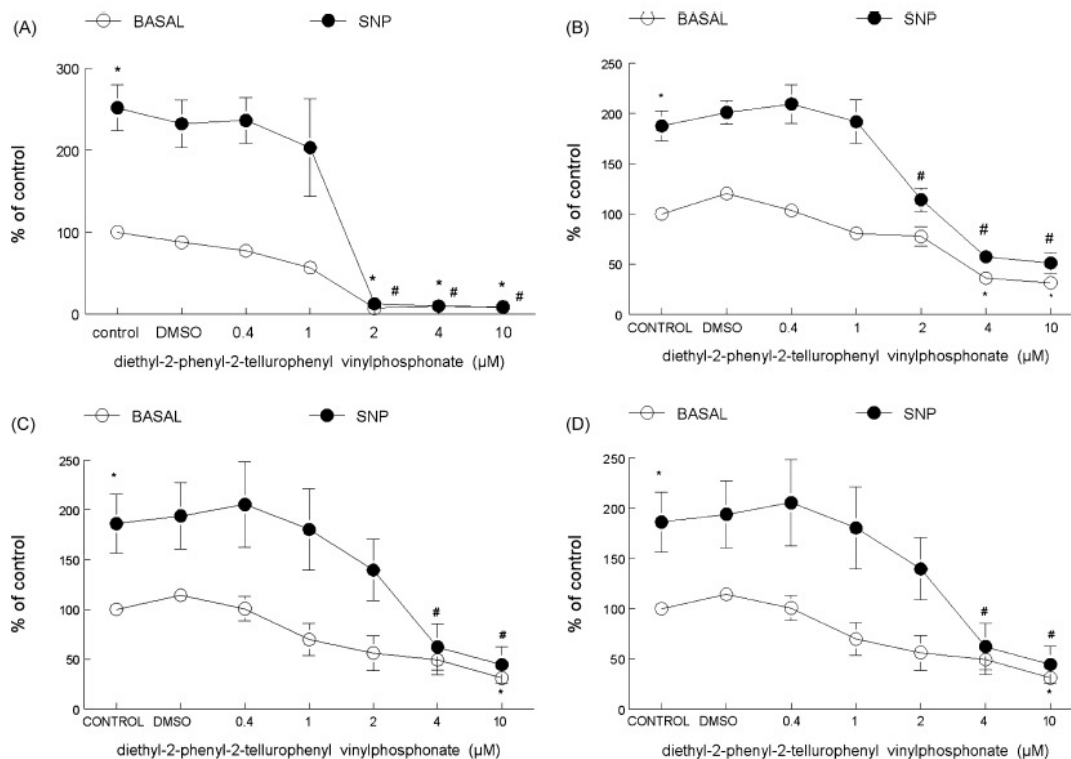

Fig. 3. Antioxidant activity of diethyl-2-phenyl-2-tellurophenyl vinylphosphonate. Spontaneous (○) and SNP-induced (●) TBARS production *in vitro* in whole brain (A), cortex (B), hippocampus (C) and striatum (D) homogenates. Results are expressed as percent of control. 100% of control group indicates 1.7 nmol MDA/g of tissue (A) and 2.4 nmol MDA/g of tissue. *Indicates statistical difference from control basal, $p < 0.05$; #Indicates statistical difference from control SNP-induced TBARS formation, $p < 0.05$, to five experiments performed in duplicates.

stimulated [^{3}H]glutamate release in comparison to control values ($p < 0.05$).

3.5. [^{3}H]Glutamate binding

The telluro vinylphosphonate did not inhibit [^{3}H]glutamate binding in membrane preparations at concentrations in which this compound exhibited antioxidant activity (lower than 10 μM). On the other hand, at the concentrations of 50 and 100 μM, [^{3}H]glutamate binding was inhibited by approximately 50% ($p < 0.05$, Fig. 7).

4. Discussion

Since the first organotellurium compounds were synthesized in 1840 by Wöhler, several studies have shown their potent antioxidant actions [14,21,75], even in brain tissue [33] and that some of them possess glutathione peroxidase (GPX)-like activity [3,22]. These studies also suggest that organotellurium compounds could act as scavengers of reactive species, making them very interesting tools in the research of antioxidant agents against the generation of reactive oxygen and/or nitrogen species in different process, including neurological disorders [36].

Our study demonstrated for the first time the antioxidant potential of telluro vinylphosphonate against QA and SNP, two chemicals that have mechanisms of action that are similar to the cascade generated by the calcium influx after glutamate binding to NMDA receptor [2]. In fact, we found different responses to the compound in the tested brain structures against the two prooxidant agents used. In hippocampus, the organotellurium compound seems to be more efficient against the QA-induced lipid peroxidation, while striatum and cortex had a similar plot from whole brain. It is known that QA may differently affect several brain regions, and that hippocampus seems to be less affected by it in some parameters, being striatum the most vulnerable region [43,80]. Besides, we could not ruled out that the oxidative effect observed for QA could be mediated by NMDA receptor overactivation, once Puntel et al. [57] demonstrated that MK-801 abolished the TBARS formation induced by QA in brain homogenates. This overactivation is associated with an increase in Ca^{2+} influx [77], ATP exhaustion and free radical production [62], leading to an increase in the lipid peroxidation both *in vitro* and *in vivo* [61,63,69]. Our results do not indicate how the telluro

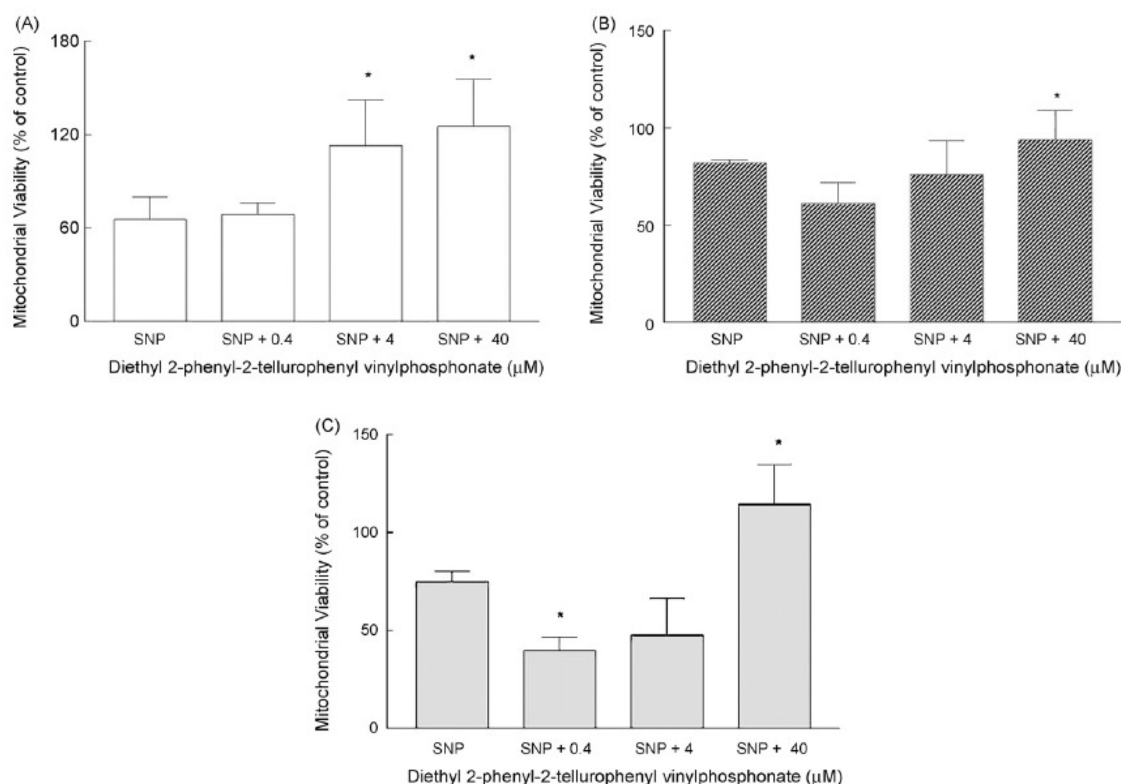

Fig. 4. Protection of decrease in mitochondrial viability induced by SNP. Different DPTVP concentrations were pre-incubated with SNP (50 μM) in cortex (A), hippocampus (B) and striatum (C). *Indicates statistical difference from SNP group of each brain region, $p < 0.05$, to three experiments.

vinylphosphonate acts against the oxidative stress induced by QA, however we can suggest that the compound possibly act as scavenger of the reactive species generated in the process, once this activity is well described to organotellurium compounds [5,14,21,31].

The same antioxidant profile was observed when SNP was used as inducer of lipid peroxidation. Therefore, the TBARS levels decreased in a more pronounced way in cortex than striatum and hippocampus. The literature describes that are some regional differences among hippocampus, cortex and striatum in relation to prooxidant and antioxidant defenses against nitric oxide damage [15,16,35]. In this cascade, the Ca^{2+} influx increases the $NO^{\bullet}$ radical formation [2,28] as well as generation of other free radicals species [10,38], especially $ONOO^{-}$, which may oxidize and covalently modify all major types of biomolecules, including DNA [29], proteins [30] and lipids [58]. Here, to mimic this process, we used SNP ($[NO–Fe–(CN)_5]$), which besides releases the $NO^{\bullet}$, generates the ferricyanide anion $[(CN)_5–Fe]^{-3}$ that can react with H_2O_2 via Fenton reaction, generating hydroxyl radicals ($OH^{\bullet}$) [27]. Several compounds have been reported in the literature as neuroprotectors due to their ability to reduce the oxidative damage caused by QA and SNP as depicted by the telluro vinylphosphonate, however few at low concentration as depicted here [63,79,56].

Mitochondrial viability is also affected by oxidative stress, once the production of reactive species causes damage to inner and outer membranes as well as the opening of the mitochondrial permeability transition pores, thereby inducing apoptosis [66]. SNP and QA are described as prooxidants agents that are able to impair mitochondrial function and decrease MTT reduction [55,49], however SNP is commonly used at high concentrations as well as QA in slices. Even the high concentration of QA (1 mM) used here to induce lipid peroxidation, similar as described by other authors [57,79], it was not capable to decrease the mitochondrial viability in the slices. This finding must be to the use of slices in this assay, once in synaptosomal fractions and cell cultures, which are more refined preparations, QA is able to cause mitochondrial dysfunction even in lower doses [19,68]. Therefore, the use of slices to observe mitochondrial viability is relevant, considering that all the constituents of the synapse are present. This paradigm is important in view that all the neuronal cells involved in a supposed exposition to the drugs *in vivo* are in the slices preparation. Besides, when QA is used in slices, it is needed a higher concentration of this prooxidant to get the expected effect [34,74].

On the other hand, we found that SNP reduced mitochondrial viability at a lower concentration than reported by other authors [8]. This effect was prevented by the antioxidant organ-

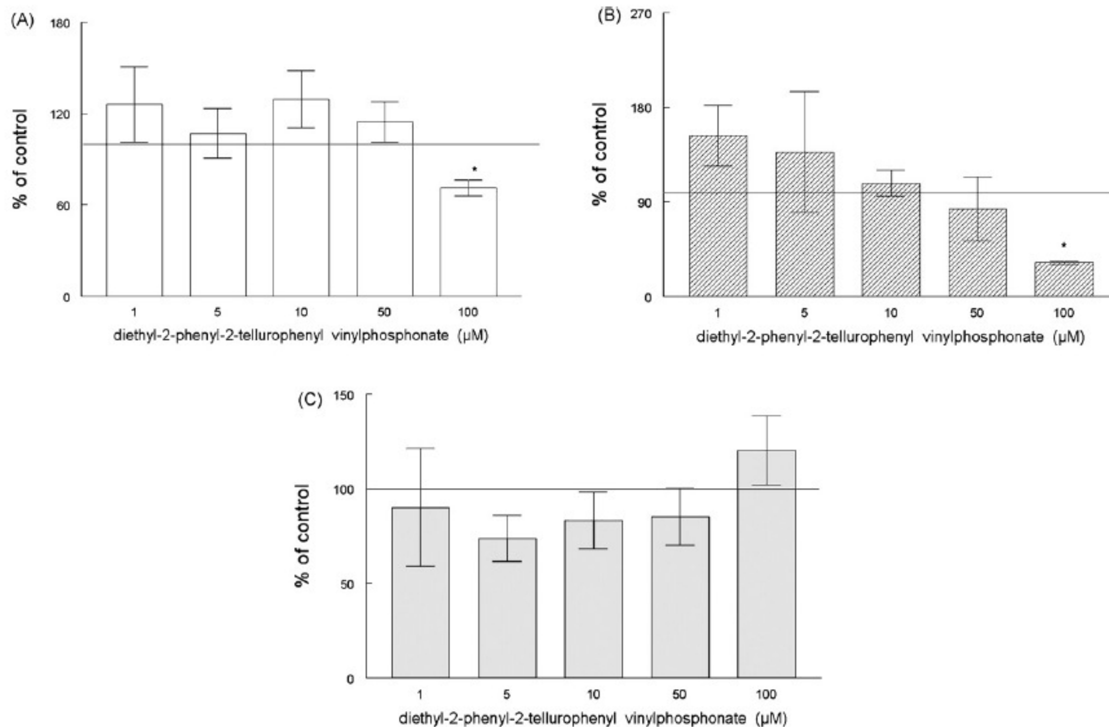

Fig. 5. Effects of diethyl-2-phenyl-2-tellurophenyl vinylphosphonate on [^{3}H]glutamate uptake. Cortex (A), hippocampus (B) and striatum (C). Data are presented as % of control ± S.E.M. 100% of control corresponds to 1.11 pmol/(mg(protein) min) (A), 1.05 pmol/(mg(protein) min) (B) and 1.02 pmol/(mg(protein) min) (C). *Indicates statistical difference from the control, $p < 0.05$ to five experiments performed in triplicates.

otellurium compound in all brain regions tested, but in a more effective way in cortex, as in TBARS assay, leading us to suggest that this brain structure is more sensitive to the antioxidant action of telluro vinylphosphonate. It is very important to emphasize that the compound did not caused mitochondrial dysfuntion at all concentrations tested here, demostrating that the organotellurium compound do not depicted to be neurotoxic *per se* in the experiments carried out here. We noted, therefore, that seems that the mitochondrial viability is diminishing with the increase of the compound concentration, nevertheless the heterogeneity of the data do not support us to believe that a dose response–curve was obtained. Of importance, the compound did not affect mitochondrial viability at the antioxidants concentrations.

Despite the antioxidants properties, it is well described that organotellurium compounds have the central nervous system as a potential target for toxic effects [52]. Some of them inhibit squalene monoxigenase [26,37] and, especially diphenyl ditteluride, inhibits Na^+/K^+ ATPase *in vitro* [12] as well as $^{45}Ca^{2+}$ influx in rat synaptosomes [46]. The glutamatergic system is also affected by the ditelluride, which causes decreases in [^{3}H]glutamate, [^{3}H] MK-801 and [^{3}H]GMP-PNP binding *in vitro* and *ex vivo* in rat membranes preparation [50] as well as decrease in [^{3}H]glutamate uptake *in vitro* in rat synaptosomes [51]. In order to assess how the telluro vinylphosphonate could affect the glutamatergic system, we evaluated the release, binding and uptake of glutamate. The [^{3}H]glutamate uptake in slices of cortex, hippocampus and striatum did not changed at the antioxidants concentrations (lower than 10 μM). When we used higher concentrations (up to 50 μM) the striatum was not affected, although a decrease in the uptake of [^{3}H]glutamate in cortex and hippocampus slices was found. In agreement, rat brain synaptosomal [^{3}H]glutamate release was altered only at 100 μM, in both basal and K^+-stimulated release. In addition, [^{3}H]glutamate binding in plasmatic membrane preparations was inhibited 50% only at the higher concentrations used here (50 and 100 μM). It is important to emphasize that in similar experiments, Nogueira et al. [51,52] observed that diphenyl ditelluride, which has similar antioxidant properties of telluro vinylphosphonate, decreased [^{3}H]glutamate uptake and [^{3}H]glutamate binding at the concentration of 10 μM, which is at least fivefold lower than that we have obtained here. This comparison is relevant, since diphenyl ditelluride is known as a good antioxidant but a very toxic agent to the central nervous system [71]. In this context, the telluro vinylphosphonate seems to be a promising organotellurium compound to be used in antioxidant neuroprotective protocols, since it could be used at high concentrations than other organotellurium compounds.

In a recent study, Ávila et al. [5] demonstrated that the telluro vinylphosphonate is able to oxidize *in vitro* –SH groups, increas-

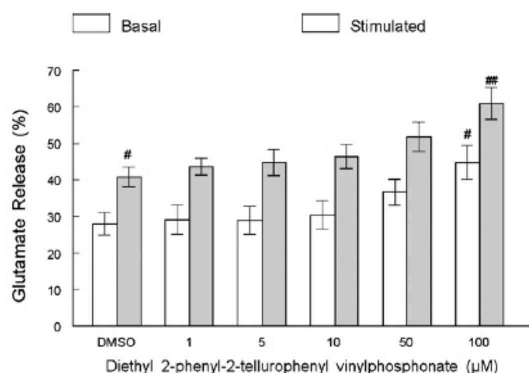

Fig. 6. Effects of diethyl-2-phenyl-2-tellurophenyl vinylphosphonate on [^{3}H]glutamate release in synaptosomal preparation. Data are expressed as % of release ± S.E.M. #Indicates statistical difference from control basal release. ##Indicates difference from control stimulated release, $p < 0.05$ to five experiments in duplicates.

ing the DTT oxidation rate and inhibiting δ-aminolevulinate dehydratase, a sulfhydryl-containing enzyme, in brain from the concentration of 120 μM. Of particular importance, the specific high-affinity EAAT and the process of glutamate exocytosis are sensitive to redox changes and also affected by oxidizing agents [25,76,81]. The EAAT contain reactive –SH groups in their structure that are modulated by their redox status [76]. Possibly, only at high concentrations the organotellurium compound or its metabolites are able to oxidize the –SH groups, resulting in the impairment in the uptake, release and binding of glutamate.

The NMDA receptor, also possess extracellular redox sites and its activity is dependent of the reduced form of the –SH groups present in their structure [41]. The NMDA receptor is partially inhibited by the –SH group oxidant DTNB (5,5′-ditio-bis-(2-nitrobenzoic acid)) and reactivated by DTT (dithiothreitol) [1,40]. Moreover, excessive glutathione extracellular levels alters the redox state of the NMDA receptor, causing neurotoxicity and an enhancement of the responses to glutamate

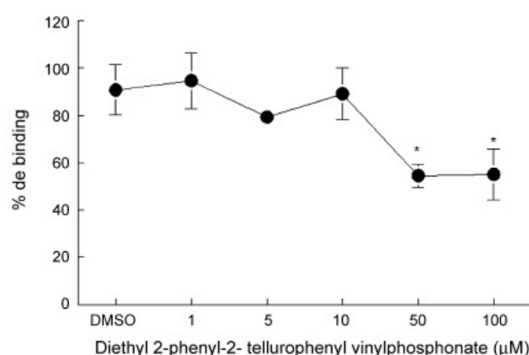

Fig. 7. Effects of diethyl-2-phenyl-2-tellurophenyl vinylphosphonate on [^{3}H]glutamate binding in membrane preparation. Data are expressed as percent of control binding ± S.E.M. *Indicates difference from control, $p < 0.05$ to five independent experiments performed in duplicate.

in neuronal cultured cells [60]. Taken together the data presented here and in previous studies [5], we can suggest that the vinylic telluride is able to oxidize these –SH groups at high concentrations, decreasing the [^{3}H]glutamate binding. It is interesting to note that NMDA –SH groups seem to be more sensitive to this compound than others sufhydryl proteins, as the glutamate transporters studied here, once the concentration necessary to inhibit the binding was lower that to decrease [^{3}H]glutamate uptake or to increase its relase. This finding could be useful in disorders related to glutamate excitotoxicity, since the organotellurium compound is able to decrease the [^{3}H]glutamate binding at 50 μM without impairment of glutamate uptake.

In conclusion, dietyl-2-phenyl-2-tellurophenyl vinylphosphonate is a potent antioxidant at low concentrations against two mimetic agents of glutamatergic excitotoxicity. This was evident by the decrement in lipid peroxidation and by the protection against mitochondrial dysfunction induced by SNP. At the antioxidant concentrations, the compound did not alter [^{3}H]glutamate uptake into brain structures slices nor its release from pre-loaded synaptosomes as well as the binding in membranes. Considering our data we can suggest that telluro vinylphosphonate: (1) presented a possible neuroprotection action due possibly to its radical scavenger activity in the brain, (2) did not alter mitochondrial function, therefore protects against SNP-induced mitochondrial dysfunction and (3) presented, at antioxidant concentrations, an absence of glutamatergic system alterations (binding, uptake and release). These properties must be explored *in vivo* to further investigate whether this organotellurium may protect against some drugs potentially toxic to the CNS and neuropathologies related to overproduction of free radicals.

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References

[1] E. Aizeman, S.A. Lipton, R.H. Loring, Selective modulation on NMDA responses by reduction and oxidation, Neuron 2 (1989) 1257–1263.
[2] A. Akaike, H. Katsuki, T. Kume, T. Maeda, Reactive oxygen species in NMDA receptor-mediated glutamate neurotoxicity, Parkinsonism Relat. Disord. 5 (1999) 203–207.
[3] C.-M. Andersson, A. Hallberg, R. Brattsand, I.A. Cotgrave, L. Engman, J. Persson, Glutathione peroxidase-like activity of diaryl tellurides, Bioorg. Med. Chem. Lett. 3 (1993) 2553–2558.
[4] R. Anwyl, Modulation of vertebrate neuronal calcium channels by neurotransmitters, Brain Res. Rev. 16 (1991) 265–281.
[5] D.S. Ávila, M.C. Beque, V. Folmer, A.L. Braga, G. Zeni, C.W. Nogueira, F.A.A. Soares, J.B.T. Rocha, Diethyl 2-phenyl-2-tellurophenyl vinylphosphonate: an organotellurium compound with low toxicity, Toxicology 224 (2006) 100–107.

[6] D.S. Ávila, P. Gubert, C.L. Dalla Corte, D. Alves, C.W. Nogueira, J.B.T. Rocha, F.A.A. Soares, A biochemical and toxicological study with diethyl-2-phenyl-2-tellurophenyl vinylphosphonate in a sub-chronic intraperitoneal treatment in mice, Life Sci. 80 (2007) 1865–1872.
[7] J.M. Barnes, J.M. Henley, Molecular characteristics of excitatory amino acid receptors, Progr. Neurobiol. 39 (1992) 113–133.
[8] S. Bastianetto, R. Quirion, Natural extracts as possible protective agents of brain aging, Neurobiol. Dis. 23 (2002) 891–897.
[9] J.N. Bates, M.T. Baker, R. Guerrra, D.G. Harrison, Nitric oxide generation from nitroprusside by vascular tissue, Biochem. Pharmacol. 42 (1991) S157–S165.
[10] J.S. Beckman, T.W. Beckman, J. Chen, P.A. Marshall, B.A. Freeman, Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide, Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 1620–1624.
[11] B. Bettler, C. Mulle, Review: neurotransmitter receptor II. AMPA and kainate receptors, Neuropharmacology 34 (1995) 123–139.
[12] V.C. Borges, J.B.T. Rocha, C.W. Nogueira, Effect of diphenyl diselenide, diphenyl ditelluride and ebselen on cerebral Na+, K+ ATPase activity in rats, Toxicology 215 (2005) 191–197.
[13] A.L. Braga, E.F. Alves, C.C. Silveira, L.H. Andrade, Stereoselective addition of sodium organyl chalcogenolates to alkynylphosphonates: synthesis of diethyl 2-(organyl)-2-(organochalcogenyl)vinylphosphonates, Tetrahedron Lett. 41 (2000) 161–163.
[14] K. Briviba, R. Tamler, L.-O. Klotz, L. Engman, I.A. Cotgreave, H. Sies, Protection against organotellurium compounds against peroxynitrite-mediated oxidation and nitration reactions, Biochem. Pharmacol. 55 (1998) 817–823.
[15] E. Candelario-Jalil, N.H. Mahdu, S.M. Al-Dalain, G. Martinez, O.S. Leon, Time-course of oxidative damage in different brain regions following transient cerebral ischemia in gerbils, Neurosci. Res. 41 (2001) 233–241.
[16] S. Cavallini, M. Marti, S. Marino, R. Selvatici, L. Beani, C. Biachi, A. Siniscalchi, Effects of chemical ischemia in cerebral córtex slices: focus on nitric oxide, Neurochem. Int. 47 (2005) 482–490.
[17] D.W. Choi, Glutamate neurotoxicity and diseases of the nervous system, Neuron 1 (1998) 623–634.
[18] N.C. Danbolt, Glutamate uptake, Progr. Neurobiol. 65 (2001) 1–105.
[19] V.P. de la Cruz, C. González-Cortés, S. Galván-Azarte, O.N. Medina-Campos, F. Pérez-Severiano, S.F. Ali, J. Pedraza-Chaverrí, A. Santamaría, Excitotoxic brain damage involves early peroxynitrite formation in a model of Huntington's disease in rats: protective role of iron porphyrinate 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinate iron (III), Neuroscience 135 (2005) 463–474.
[20] P.R. Dunkley, P.E. Jarvie, J.W. Heath, G.J. Kidd, J.A.P. Rostas, A rapid method for isolation of synaptosomes on Percoll gradients, Brain Res. 372 (1986) 115–129.
[21] L. Engman, J. Person, K. Vessman, M. Ekstrom, M. Berglund, C.-M. Andersson, Organotellurium compounds as efficient retarders of lipid peroxidation in methanol, Free Rad. Biol. Med. 19 (1995) 441–452.
[22] L. Engman, D. Stern, I. Cotgreave, C.-M. Andersson, Thiol peroxidase activity of diaryl ditellurides as determined by a ^{1}H NMR method, J. Am. Chem. Soc. 114 (1992) 9737–9743.
[23] M.E.S. Frizzo, D.R. Lara, A.S. Prokopiuk, C.R. Vargas, C.G. Salbego, M. Wajner, D.O. Souza, Guanosine enhances glutamate uptake in brain cortical slices at normal and excitotoxic conditions, Cell Mol. Neurobiol. 22 (2002) 353–363.
[24] E.M. Fykse, F. Fonnum, Amino acid neurotransmission: dynamics of vesicular uptake, Neurochem. Res. 21 (1996) 1053–1060.
[25] S.C. Gilman, M.J. Bonner, T.C. Pellmar, Peroxide effects on [^{3}H]glutamate release by synaptosomes isolated from cerebral cortex, Neurosci. Lett. 22 (1992) 157–160.
[26] J.F. Goodrum, Role of organotellurium species in tellurium neuropathy, Neurochem. Res. 10 (1998) 1313–1319.
[27] G. Graf, J.R. Mahoney, R.G. Bryant, J.W. Eaton, Iron-catalyzed hydroxyl radical formation-stringent requirement for free iron coordination site, J. Biol. Chem. 259 (1984) 3620–3624.
[28] C. Iadecola, Bright and dark sides of nitric oxide in ischemic brain injury, Trends Neurosci. 20 (1997) 132–139.
[29] S. Inoue, S. Kawanish, Oxidative DNA damage induced by simultaneous generation of nitric oxide and superoxide, FEBS Lett. 371 (1995) 86–88.
[30] H. Ischiropoulos, A.B. Al-Mehdi, Peroxynitrite mediated oxidative protein modifications, FEBS Lett. 364 (1995) 279–282.
[31] C. Jacob, G.E. Arteel, T. Kanda, L. Engman, H. Sies, Water soluble organotellurium compounds: catalytic protection against peroxynitrite and release of zinc from metallothionein, Chem. Res. Toxicol. 13 (2000) 3–9.
[32] D.H. Jones, A.I. Matus, Isolation of synaptic plasma membrane from brain by combined flotation–sedimentation density gradient centrifugation, Biochim. Biophys. Acta 356 (1974) 276–287.
[33] J. Kanski, J. Drake, M. Aksenova, L. Engman, D.A. Butterfield, Antioxidant activity of the organotellurium compound 3-[4-(*N,N*-dimethylamino)benzenetellurenyl]propanesulfonic acid against oxidative stress in synaptossomal membrane systems and neuronal cultures, Brain Res. 911 (2001) 12–21.
[34] H. Katsuki, A. Akaike, Excitotoxic degeneration of hypothalamic orexin neurons in slice culture, Neurobiol. Dis. 15 (2004) 61–69.
[35] J.Y. Khan, S.M. Black, Developmental changes in murine brain antioxidant enzyme, Pediatric. Res. 54 (2003) 77–82.
[36] J. Krieglstein, Excitotoxicity and neuroprotection, J. Pharm. Sci. 5 (1996) 181–187.
[37] B.P. Laden, T.D. Porter, Inhibition oh human squalene monooxigenase by tellurium compounds: evidence of interaction with vicinal sulfhydryls, J. Lipid Res. 42 (2001) 235–340.
[38] M. Lafon-Cazal, M. Culcasi, F. Gaven, S. Pietri, J. Bockeart, Nitric oxide, superoxide and peroxynitrite: putative mediators of NMDA-induced cell death in cerebellar granule cells, Neuropharmacology 32 (1993) 1259–1266.
[39] O.R. Leeuwenkamp, N.L.J. Chin, E.J. Van der Mark, W.P. Van Bennekom, A. Bult, *In vitro* degradation of nitroprusside in relation to *in vivo* decomposition and mechanism of action, Int. J. Pharm. 33 (1986) 1–13.
[40] S.Z. Lei, Z.H. Pan, S.K. Aggarwal, H.S.V. Chen, S.A. Lipton, Effect of nitric oxide production on the redox modulatory site of NMDA receptor-channel complex, Neuron 8 (1992) 1087–1099.
[41] S.A. Lipton, Y.B. Choi, Z.H. Pan, S.Z. Lei, H.S.V. Chen, N.J. Sucher, D.J. Singel, J.S. Stampler, A redox state mechanism for neuroprotective and neurodestructive effects of nitric oxide and nitroso compounds, Nature 364 (1993) 626–632.
[42] O.H. Lowry, N.J. Rosebrough, A. Lewis-Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[43] K. Maeda, H. Kaneda, W.O. Whetsell Jr., C.A. Tamminga, Neurochemical and metabolic consequences of elevated cerebrospinal fluid quinolinic acid concentrations in rat brain, Neurosci. Res. 29 (1997) 303–309.
[44] B.S. Meldrum, Glutamate as a neurotransmitter in the brain: review of physiology and pathology, J. Nutr. 130 (2000) 1007S–1015S.
[45] P.V. Migues, R.B. Leal, M. Mantovani, M. Nicolau, N.H. Gabilan, Synaptosomal glutamate release induced by the fraction BC2 from the venom of the sea anemone Bunadosoma caissarum, Neuroreport 10 (1999) 67–70.
[46] M.B. Moretto, J.I. Rossato, C.W. Nogueira, G. Zeni, J.B.T. Rocha, Voltage-dependent ebselen and diorganochalcogenides inhibition of $^{45}Ca^{2+}$ influx into brain synaptosomes, J. Biochem. Mol. Toxicol. 17 (2003) 154–160.
[47] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay, J. Immunol. Methods 16 (1983) 55–63.
[48] M. Nedergaard, T. Takano, A.J. Hansen, Beyond the role of glutamate as a neurotransmitter, Neuroscience 3 (2002) 748–755.
[49] B.-M. Nie, L.-M. Yang, S.-L. Fu, X.-Y. Jiang, P.-H. Xu, Y. Lu, Protective effect of panaxydol and panaxynol on sodium nitroprusside-induced apoptosis in cortical neurons, Chem. Biol. Interact. 160 (2006) 225–231.
[50] C.W. Nogueira, L.N. Rotta, M.L. Perry, D.O. Souza, J.B.T. Rocha, Diphenyl diselenide and diphenyl diteluride affect the rat glutamatergic system in vitro and in vivo, Brain Res. 906 (2001) 157–163.
[51] C.W. Nogueira, L.N. Rotta, G. Zeni, D.O. Souza, J.B.T. Rocha, Exposure to ebselen changes glutamate uptake and release by rat brain synaptossomes, Neurochem. Res. 27 (2002) 283–288.
[52] C.W. Nogueira, G. Zeni, J.B.T. Rocha, Organoselenium and organotellurium compounds: toxicology and pharmacology, Chem. Rev. 104 (2004) 6255–6285.

[53] H. Ohkawa, N. Ohishi, K. Yagi, Assay for lipid peroxides in animal tissues bu thiobarbituric acid reaction, Anal. Biochem. 95 (1979) 351–358.

[54] S. Ozawa, H. Kamiya, K. Tsuzuki, Glutamate receptors in the mammalian central nervous system, Prog. Neurobiol. 54 (1998) 581–618.

[55] V. Pérez-de La Cruz, C. González-Cortéz, S. Galván-Arzate, O.N. Medina-Campos, F. Pérez-Severiano, S.F. Ali, J. Pedraza-Chaverri, A. Santamaría, Excitotoxic brain damage involves early peroxynitrite formation in a model of Huntington's disease in rats: protective role of iron porphyrinate 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinate iron (III), Neuroscience 135 (2005) 463–474.

[56] T. Posser, M.B. Moretto, A.L. Dafre, M. Farina, J.B.T. Rocha, C.W. Nogueira, G. Zeni, J.S. Ferreira, R.B. Leal, J.L. Franco, Antioxidant effect od diphenyl diselenide against sodium nitroprusside (SNP) induced lipid peroxidation in human platelets and erythrocyte membranes: an in vitro evaluation, Chem. Biol. Interact. 164 (2006) 126–135.

[57] R. Puntel, C.W. Nogueira, J.B.T. Rocha, *N*-Methyl-D-aspartate receptors are involved in the quinolinic acid, but not in the malonate pro-oxidative activity *in vitro*, Neurochem. Res. 30 (2005) 417–424.

[58] R. Radi, J.S. Beckman, K.M. Bush, B.A. Freeman, Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide, Arch. Biochem. Biophys. 288 (1991) 481–487.

[59] P. Rauhala, A. Khaldi, K.P. Mohanakumar, C.C. Chiueh, Apparent role of hydroxyl radicals on oxidative brain injury induced by sodium-nitropruside, Free Rad. Biol. Med. 24 (1998) 1065–1073.

[60] R.F. Regan, Y.P. Guo, Potentiation of excitotoxic injury by high concentrations of extracellular reduced glutathione, Neuroscience 91 (1993) 463–470.

[61] C. Rios, A. Santamaria, Quinolinic acid is a potent lipid peroxidant in rat brain homogenates, Neurochem. Res. 16 (1991) 1139–1141.

[62] E. Rodriguez-Martinez, A. Camacho, P.D. Maldonado, J. Pedraza-Chaverrí, D. Santamaría, S. Galván-Arzate, A. Santamaría, Effect of quinolinic acid on endogenous antioxidants in rat corpus striatum, Brain Res. 858 (2000) 436–439.

[63] J.I. Rossato, G. Zeni, C.F. Mello, M.A. Rubin, J.B.T. Rocha, Ebselen blocks the quinolinic acid-induced production of thiobarbituric acid reactive species but does not prevent the behavioral alterations produced by intrastriatal quinolinic acid administration in the rat, Neurosci. Lett. 318 (2002) 137–140.

[64] A. Santamaría, A. Flores-Escartin, J.C. Martinez, L. Osorio, S. Galvan-Arzate, J. Pedraza-Chaverri, P.D. Maldonado, O.N. Medina-Campos, M.E. Jimenez-Capdeville, J. Manjarrez, C. Rios, Copper blocks quinolinic acid neurotoxicity in rats: contribution of antioxidant systems, Free Rad. Biol. Med. 35 (2003) 418–427.

[65] A. Santamaría, C. Rios, MK-801, an *N*-methyl-D-aspartate receptor antagonist, blocks quinolinic acid-induced lipid peroxidation in rat corpus striatum, Neurosci. Lett. 159 (1993) 51–54.

[66] K. Sas, H. Robotka, J. Toldi, L. Vécsei, Mitochondria, metabolit disturbances, oxidative stress and kynurenine system, with focus on neurodegenerative disorders, J. Neurol. Sci. 257 (2007) 221–239.

[67] T.W. Scalbert, Neuropharmacology of quinolinic acid in kynurenic acids, Pharmacol. Rev. 45 (1993) 309–379.

[68] P.F. Schuck, A. Tonin, G.C. Ferreira, R.B. Rosa, A. Latini, F. Balestro, M.L.S. Perry, C.M.D. Wannamacher, A.T.S. Wyse, M. Wajner, In vitro effect of quinolinic acid on energy metabolism of young rats, Neurosci. Res. 57 (2007) 277–288.

[69] R. Schwarcz, A.C. Foster, E.D. French, W.O. Whetsell Jr., C. Kohler, Excitotoxic models for neurodegenerative disorders, Life Sci. 95 (1984) 29–38.

[70] F.A. Soares, M. Farina, F.W. Santos, D. Souza, J.B.T. Rocha, C.W. Nogueira, Interaction between metal and chelating agents affects glutamate binding on brain synaptossomal membranes, Neurochem. Res. 28 (2003) 1859–1865.

[71] E.C. Stangherlin, A.M. Favero, G. Zeni, J.B.T. Rocha, C.W. Nogueira, Exposure of mothers to diphenyl ditelluride during the suckling period changes behavior tendencies in their offspring, Brain Res. Bull. 69 (2006) 311–317.

[72] T.W. Stone, Kynurenine and glycine enhance neuronal sensitivity by *N*-methyl-D-aspartate, Life Sci. 48 (1991) 765–772.

[73] S. Takamori, VGLUTs: "Exciting" times for glutamatergic research? Neurosci. Res. 55 (2006) 343–351.

[74] M.T. Tebano, M.R. Domenici, P. Propoli, SCH 58261 differentially influences quinolinic acid induced effects in striatal and in hippocampal slices, Eur. J. Pharmacol. 450 (2002) 253–257.

[75] L. Tiano, D. Fedeli, A.M. Santroni, M. Villarini, L. Engman, G. Falcioni, Effect of three diaryl tellurides, and organoselenium compound in trout erythrocytes exposed to oxidative stress in vitro, Gen. Toxicol. Environ. Mutat. 464 (2000) 269–277.

[76] D. Trotti, B.L. Rizzini, D. Rossi, O. Haugeto, G. Racagni, N.C. Danbolt, A. Volterra, Neuronal and glial glutamate transporters possess a SH-based redox regulatory mechanism, Eur. J. Neurosci. 9 (1999) 1236–1243.

[77] K. Tsuzuki, M. Lino, S. Ozawa, Change in calcium permeability caused by quinolinic acid in culture rat hippocampal neurons, Neurosci. Lett. 105 (1989) 269–274.

[78] M.G. Vegh, A.D. Kovacs, G. Szabo, K. Tihanyi, L.G. Harsing Jr., G. Levay, The new 2,3-benzodiazepine derivatives EGIS-8332 inhibits AMPA/kainite ion channels and cell death, Neurochem. Int. 50 (2007) 553–563.

[79] C. Wagner, R. Fachinetto, C.L. Dalla Corte, V.B. Brito, D. Severo, G.O. Dias, A.F. Morel, C.W. Nogueira, J.B.T. Rocha, Quercetrin, a glycoside form of quercetin, prevents lipid peroxidation in vitro, Brain Res. 1107 (2006) 192–198.

[80] W.O. Whetsell Jr., R. Schwarcz, Prolonged exposure to sub-micromolar concentrations of quinolinic acid causes excitotoxic damage in organotypic cultures of rat corticostriatal system, Neurosci. Lett. 97 (1989) 271–275.

[81] F. Zoccarato, L. Cavallini, M. Valente, A. Alexandre, Modulation of glutamate exocytosis by redox agents of superficial thiol groups in rat cerebrocortical synaptosomes, Neurosci. Lett. 274 (1999) 107–110.

IV.2. POSSÍVEL AÇÃO NEUROPROTETORA DE UM TELURETO VINÍLICO CONTRA A NEUROTOXICIDADE INDUZIDA POR MANGANÊS

Manuscrito 1

A POSSIBLE NEUROPROTECTIVE ACTION OF A VINYLIC TELLURIDE AGAINST MN-INDUCED NEUROTOXICITY

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Em fase de redação

A possible neuroprotective action of a vinylic telluride against Mn-induced neurotoxicity

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Abstract

Manganese (Mn) is a required metal for biological systems, nevertheless environmental or occupational exposure to Mn has been recognized to produce a neurological disorder with similarities to Parkinson's disease (manganism). Diethyl-2-phenyl-2-tellurophenyl vinylphosphonate (DPTVP) is an organotellurium compound with a high antioxidant activity and hepatoprotector activity. Our study was designed to investigate the effects of a long - term Mn exposure in mild doses in the drinking water on some behavioral and biochemical parameters in Wistar rats and whether the vinylic telluride could be able to revert the alterations. After 4 months of treatment with $MnCl_2$ (6.9mg/ml in the drinking water) rats exhibited clear signs of neurobehavioral toxicity including a decrease in the number of crossings and rearings in the open field task and a decrease in the latency to the first fall in the rotarod test. The administration of DPTVP (0.150µmol/Kg, i.p., 2 weeks) restored the decreased number of crossings and the latency for the first fall. We detected that Mn-treated rats`striatum showed higher susceptibility to Mn than cortex and hippocampus, once the mitochondrial viability and [^{3}H]glutamate uptake were significantly decreased and lipid peroxidation was increased only in striatum. These results were in accordance with the high Mn levels detected in striatum of the Mn-treated rats. The treatment with the telluride recovered the mitochondrial damage, TBARS levels and the [^{3}H]glutamate uptake to control levels. Furthermore, DPTVP seems to interfere with Mn influx/effflx in the brain, once we have found decreased levels of Mn in the striatum of DPTVP-treated rats. Our results demonstrated that Mn exposure may cause serious biochemical alterations in striatum and consequently in the locomotor activity of Mn-treated rats and that the vinylic telluride has a neuroprotector activity due its antioxidant activity and/or by altering Mn transport in striatum.

Keywords: tellurim, manganese neurotoxicity, oxidative stress, glutamate, mitochondria, neuroprotector.

Introduction

Chronic environmental or occupational exposure to Mn has been recognized to produce a parkinsonian or dystonic state in mammals, so-called manganism (Barbeau, 1984; Wang *et al.*, 1989). Especial concern has been rising about the chronic exposure to Mn once it is present in a gasoline additive (methylcyclopentadienyl manganese tricarbonyl), contaminating the air and water sources (Hinderer, 1979). Mn exposure can cause motor effects in mammals that are described as extrapyramidal anomalies, such as rigidity, hipokinesia, tremor and gait disturbances (Newland, 1999). Such effects have been attributed to a selective interaction of Mn with the basal ganglia downstream of the nigrostriatal dopaminergic projection, in particular with the globus pallidus and striatum (Sloot e Gramsbergen, 1994; Shinotoh *et al.*, 1997). Nevertheless, it has been reported that Mn neurotoxicity may be due to an indirect excitotoxic event caused by altered glutamate metabolism, which could be primary to the dopaminergic effects (Brouillet *et al.*, 1993).

Studies have demonstrated that astrocytes can accumulate Mn by a high affinity, capacity, and specific transport system (Aschner *et al.*, 1992), with a major consequence of exposure to this metal being the development of Alzheimer type II astrocytosis (Pentschew *et al.*, 1963). In this condition, the astrocytes show morphological and metabolic alterations, leading to decreased function of these glial cells (Cavanagh e Kyu, 1971). As the glutamate uptake predominantly occurs in astrocytes (Danbolt, 2001), this function is impaired by Mn exposure, as already demonstrated in vitro (Hazell e Norenberg, 1997). Furthermore,(Erikson e Aschner, 2002) have shown that the decrease in glutamate uptake by Mn involves the decrease in the mRNA of the major glutamate transporter in astrocytes, the glutamate/aspartate transporter (GLAST). This causes an abnormal increase in the glutamate levels in the synaptic cleft, which is excitotoxic to neurons (Choi, 1988).

It is still unknown how Mn exposure can lead to altered glutamatergic transmission, however the current hypothesis considers the impairment of energy metabolism resulting

from mitochondrial dysfunction and free radical production (Verity, 1999; Erikson *et al.*, 2004). At the cellular level, Mn accumulates in mitochondria (Wedler *et al.*, 1989) and inhibits the complexes of the electron transport chain (Zhang *et al.*, 2004), impairing the oxidative phosphorylation (Gavin *et al.*, 1992) and the ATP production (Brouillet *et al.*, 1993). The decreased energy production alters the mitochondrial permeability transition, causing excessive Ca^{2+} influx and massive reactive oxygen species (ROS) production (Rao e Norenberg, 2004). The ROS cause oxidation of important cellular components, such as lipids (Halliwell e Chirico, 1993), as well as alterations in the excitatory transmission, in particular by causing abnormal glutamate release and defective glutamate uptake (Greene e Greenamyre, 1996; Danbolt, 2001).

As oxidative stress seems to play a central role in Mn- induced neurotoxicity, several authors have investigated the potential neuroprotective effect of antioxidants against Mn toxicity. In mitochondrial preparations, N-acetylcysteine (NAC), glutathione (GSH) and Vitamin C prevented ROS production caused by high concentrations of Mn (Zhang *et al.*, 2004). In cultured astrocytes, Chen and Liao (Chen e Liao, 2002) observed that NAC attenuated the pro-oxidant effects of Mn. Ex vivo, Hazell et al reported that NAC blocked the astrocytosis caused by acute exposure to Mn (Hazell *et al.*, 2006).

Organotellurium compounds are potent in vitro antioxidants (Engman *et al.*, 1995; Wieslander *et al.*, 1998; Souza *et al.*, 2009a). Nevertheless, they can also be extremely toxic after in vivo exposure (Toews *et al.*, 1997; Laden e Porter, 2001; Nogueira, Zeni *et al.*, 2004). Despite this we have recently observed that the nature of the organic moiety can influence considerably the toxicity of organotellurides in rodents (Savegnago *et al.*, 2006; Borges *et al.*, 2008). The diethyl-2-phenyl-2-tellurophenyl vinylphosphonate as a very low toxic vinylic telluride compound (LD_{50} to mice > 500µmol/Kg and to rats ~4µmol/Kg), even to the brain (Avila *et al.*, 2007; Avila *et al.*, 2008). Furthermore, this compound has a high antioxidant activity (De Avila *et al.*, 2006; Avila *et al.*, 2007; Avila *et al.*, 2008) and was reported as hepatoprotector in a model in which the oxidative stress is strongly involved (Avila, D. S. *et al.*, 2009). As already depicted here, Mn neurotoxicity involves intense oxidative stress and has been attracting environmental

attention. Hence, our aim in this study was to investigate the effects in rats of a long - term Mn exposure I) in their motor activity *in vivo*; II) in the biochemical parameters in different brain areas *ex-vivo*; III) and whether the vinylic telluride could be able to revert both behavior and biochemical alterations due to its antioxidant activity. For this purpose, we evaluated the animals in the open field and rotarod task and measured the lipid peroxidation, mitochondrial viability, ROS production, superoxide dismutase activity and [^{3}H]glutamate uptake in cortex, hippocampus and striatum of rats, once no previos study has investigated simultaneously these supposed important end-poinst of Mn neurotoxicity after in vivo exposure.

2. Materials and Methods

2.1. Chemicals

Diethyl 2- phenyl-2-tellurophenyl vinylphosphonate (Figure 1) was synthesized by the addition of alkynylphosphonates to a solution of sodium organyl tellurolate, prepared by the reduction of diorganyl ditellurides with sodium borohydride in ethanol at room temperature (Braga *et al.*, 2000a). Manganese chloride ($MnCl_2.H_2O$ ~99% of purity) and all other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.2. Animals

Adult Wistar male rats (200-250g) from our own breeding colony were maintained in an air conditioned room (20–25℃) under natural lighting conditions with water and food (Guabi-RS, Brasil) *ad libitum.* All experiments were conducted in accordance with the Guiding Principles of the Animal Care and Wellness Committee of the Universidade Federal de Santa Maria.

2.3. Animal's treatment

Twenty animals were divided into two groups with 10 animals each. Group 1 received daily for four months only drinking water; group 2 received for the first 15 days 50mg/Kg of $MnCl_2$ dissolved in the drinking water daily for habituation and after 137 mg/Kg of $MnCl_2$ for the rest of the period of the treatment. The Mn dose was chosen based in previous pilot experiments which we observed behavioral alterations characteristic of Mn neurotoxicity with this dose after 4 months of oral exposure.

These animals were monitored by drinking water volume every two days (data not shown) and by gain/loss weight and behavioral alterations monthly. When the behavioral alterations were observed in the animals from the group 2, we divided both group 1 and 2 in another 2 groups as follows, with 5 animals each one:

1- Control (water/vehicle (canola oil, i.p.);
2- DPTVP control (water/DPTVP 0.150µmol/Kg, i.p.)
3- Mn ($MnCl_2$/ canola oil i.p.);
4- Mn+DPTVP (MnCl2/ DPTVP 0.150µmol/Kg, i.p.)

The rats received the i.p. injections of vehicle or DPTVP for two weeks concomitantly to the treatment in the drinking water and were maintained under behavioral monitoration.

2.4. Behavioral Evaluations

2.4.1 Open Field

Animals were individually placed at the center of the open field apparatus (45 cmX45 cmX30 cm, divided into 9 squares). Spontaneous ambulation (number of segments crossed with the four paws) and exploratory activity (expressed by the number of rearings on the hind limbs) were recorded for 6 min (Burger *et al.*, 2005).

2.4.2 Rotarod task

The integrity of motor system was evaluated using the rotarod test. Briefly, the rotarod apparatus consists of a rod 30cm long and 3cm in diameter that is subdivided

into three compartments by discs 24cm in diameter. The rod rotates at a constant speed of 10rpm. The latency for first fall off from the rod and number of falls were noted. The cut-off time was 120s.

2.5. Biochemical Analysis

2.5.1. Tissue preparation

At the end of the treatment, rats were euthanized, the brain was removed and the cortex, the two hippocampi and two striata were dissected. Half of the cortex, one hippocampus and one striatum were homogenized (1:10) in 10mM Tris- buffer (pH 7.4) and another half of cortex, one hippocampus and one striatum were cut in slices (0.4mm) in a McIlwain chopper to be used in the biochemical assays.

2.5.2 Thiobarbituric acid reactive substances production

Two slices of cortex, hippocampus and striatum were homogenized in ultra-purified water, and then the TBA reagent (15% of trichloroacetic acid, 0.375% of thiobarbituric acid and 2.5% v/v of HCl) was added. After 30 min of incubation at 100˚C, samples were centrifuged (3,000xg, 15 min) and then TBARS levels were measured at 532 nm (Rios e Santamaria, 1991). An aliquot of the homogenate was used for protein determination.

2.5.3 Mitochondrial viability

Neuronal injury in cortex, hippocampus and striatum was quantified by measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a dark violet formazan product by mitochondrial dehydrogenases (Mosmann, 1983). Slices of the brain areas were pre-incubated at 37 ◦C for 15 minutes in 500 µL of oxygenated buffer, containing (in mM): 118 NaCl, 1.2 KH_2PO_4, 4.7 KCl, 2.5 $CaCl_2$, and 1.17 $MgSO_4$. Then, the MTT reduction assay was started by adding 0.5 mg/mL MTT. After 60 min of incubation, medium was removed and the the formazan in the slices was solubilized in 1.5mL dimethylsulfoxide (DMSO). The rate of MTT reduction was measured spectrophotometrically in the supernatant at a test wavelength of 570 nm and a reference

wavelength of 630 nm. The slices were solubilized in SDS 1% and an aliquot was used for protein determination.

2.5.4 [^{3}H]Glutamate uptake

Slices were pre-incubated at 35 ◦C for 15 min, and then washed with a HBSS solution containing (mM): 137 NaCl, 0.63 Na_2HPO_4, 4.17$NaHCO_3$, 5.36 KCl, 0.44 KH_2PO_4, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$ and 5.55 glucose, adjusted to pH 7.2. The glutamate uptake was performed according to Frizzo et al. (Frizzo *et al.*, 2002) with some modifications. Briefly, uptake was carried out at 35 ◦C by adding 100µM of unlabeled glutamate and 0.33µCi/mL [^{3}H]glutamate. The reaction was stopped after 7 (cortex), 5 (hippocampus) and 3 (striatum) minute by washing two times with 1mL cold HBSS, immediately followed by addition of 0.5N NaOH, which was kept overnight. Na independent uptake was determined by using choline chloride instead of NaCl, which was subtracted from the total uptake to obtain the Na^+-dependent uptake. Incorporated radioactivity was determined with a Packard scintilator (TRI CARB 2100 TR). All experiments were performed in triplicate.

2.5.5 Superoxide dismutase activity

SOD was determined in S1 of cortex, hippocampus and striatum according to the methodology of Misra and Fridowich (1972) (Misra e Fridovich, 1972). The adrenochrome production was measured spectrophotometrically at 480 nm. One unit of the enzyme was defined as the amount of enzyme required to inhibit the rate of adrenaline auto-oxidation by 50%.

2.5.6 Estimation of ROS generation

Generation of ROS was estimated with the fluorescent probe, 2',7'-dichlorofluorescein diacetate (DCFH-DA), as described by Ali et al. (1992) (Ali *et al.*, 1992). Briefly, 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-DA (5 µM) at 37 C for 60 min.

The DCFH-DA is enzymatically hydrolyzed by intracellular esterases to form nonfluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS. DCF fluorescence intensity is proportional to the amount of ROS that is formed. Fluorescence was measured using excitation and emission wavelengths of 480 and 535 nm, respectively. A calibration curve was established with standard DCF (0.1 nM to 1 µM), and ROS levels were expressed as percentages of control.

2.5.7 Measurement of Mn levels

Total Mn content was determined in an analytical chemistry lab of the Universidade Federal de Santa Maria. The striatum samples were digested in 2mL concentrated nitric acid, with 0.5mL hydrogen peroxide added. Total Mn in the samples was determined using a flameless graphite furnace atomic absorption spectrometry.

2.5.8 Protein determination

Aliquots from the homogenized slices and homogenates were separated to protein measurements that were assessed according to Lowry et al. (1951) (Lowry *et al.*, 1951).

Statistical Analysis

Statistical significance was assessed by MANOVA (for repeated measures) or by one-way ANOVA, followed by Student-Newman-Keuls (SNK) test for post hoc comparison. Results were considered statistically significant when $p<0.05$.

3. Results

3.1 Behavioral evaluations

After 4 months of treatment with $MnCl_2$, we observed that rats showed no changes in the body weight (Table 1), nevertheless we noted alterations in the behavioral tests, such as decrease in the number of rearings in the open field, decrease in the latency to the first fall and increase in the number of falls in the rota rod task ($p<0.05$, Table 2). The

administration for of DPTVP for two weeks (0.150µmol/Kg, i.p.) improved the alterations in the open field task by revert the decreased number of rearings ($p<0.05$, Figure 1B). No significant effect was observed in the number of crossings (Figure 1A). Furthermore the vinylic telluride increased the latency to the first fall in the rotarod task that was decreased by Mn exposure ($p<0.05$, Figure 1C). No improvement was observed in the number of falls (data not shown).

3.2 Biochemical analysis

In the figure 2 we can observe that Mn exposure caused increase in TBARS production only in striatum ($p<0.05$, Fig. 2C) and not in cortex and hippocampus (Fig. 2B-C). Sub- chronic treatment with DPTVP caused decrease in the lipid peroxidation to the control levels in striatum (Figure 2C). On the other hand, when we evaluated the ROS formation using the DCF dye, we did not observe any difference among the groups in any of the analyzed structures (data not shown). SOD activity was slightly decreased by Mn in hippocampus and striatum, but the results were not statistically significant (Table 3).

As previously described by other authors, we detected that mitochondrial viability was decreased by Mn exposure, and only in striatum ($p<0.05$, Fig. 3C). Cortical and hippocampal MTT reduction were not affected by Mn. The treatment with the telluride recovered the mitochondria from the damage ($p<0.05$, Fig. 3C).

Similarly, [^{3}H]glutamate uptake was decreased by Mn treatment in striatum ($p<0.05$, Fig 4C) but no alterations were observed in cortex and hippocampus (Fig. 4A-B). DPTVP administration recovered the [^{3}H]glutamate uptake to the control levels ($p<0.05$, Fig 4C).

In order to explain the selective striata toxicity, Mn levels were quantified in this brain structure. Corroborating to our results, we found high Mn levels only in the striatum of Mn-treated animals in comparison to control ones ($p<0.05$, Figure 5). Striatum from control animals contained 0.26±0.06 µg of Mn/g of tissue while Mn-treated ones contained 0.52±0.16 µg/g. Furthermore, the animals treated with DPTVP showed Mn levels similar to the controls ones (0.204±0.027µg/g) ($p<0.05$, Figure 5).

Discussion

Our current investigation demonstrated for the first time a neuroprotective action of the diethyl-2-phenyl-2-tellurophenyl vinylphosphonate against Mn-induced neurotoxicity. We used a rat model exposure with mild doses of the metal in the drinking water, mimicking an environmental chronic exposure. We observed that Mn exposure caused significant behavioral alterations associated with increase in oxidative stress and alterations in the glutamatergic neurotransmission in striatum, which were almost completely recovered by the post-treatment for only 14 days with the vinylic telluride.

Exploratory and locomotor levels measured in an open field have been widely used in behavioral studies. The open field procedure involves the evaluation of the primary motor activity. Previous studies have shown that Mn exposure can increase (St-Pierre *et al.*, 2001), decrease (Cano *et al.*, 1997) or not modify activity levels, depending on the route of administration, the dose and the period of exposure (Dorman *et al.*, 2000). Here, we observed decreased motor and exploratory activities in Mn-treated rats after a long term treatment with mild doses of the metal. Our work demonstrates that oxidative stress and the glutamatergic system could be involved in these behavioral changes induced by Mn, once the treatment with this metal also caused increase in TBARS levels and decrease in mitochondrial viability and in [^{3}H]glutamate uptake. Furthermore, we observed that DPTVP recovered the motor control of the treated rats, as indicated by the increased latency to the first fall in comparison to Mn-treated animals, as well as improved their exploratory activity, as indicated by the number of rearings at the control values. We believe that this improvement is associated to the antioxidant activity depicted by DPTVP and/or to the ability of the compound to counteract with Mn brain transport. Less Mn in the rats`striatum could decrease its effects on the dopaminergic and even glutamatergic systems, but we believe that at least a vestige of the Mn effects should be observed. Nevertheless, the antioxidant capacity of the DPTVP improved the damage caused by Mn in the striatum, contributing to the recovery of the motor activity.

Several lines of evidence indicate that intracellularly, Mn accumulates (~100nM) within the mitochondria (Gavin *et al.*, 1992; Malecki, 2001), and thereby collapses the

mitochondrial membrane potential, produces bioenergetic defect and enhances the generation of free radicals (Gavin *et al.*, 1992). Here we confirmed such damage, once Mn decreased mitochondrial viability and increased lipid peroxidation in striatum. We expected to observe also an increase in the ROS levels and alteration in the SOD activity, but we believe that as the animals were exposed chronically to Mn we just observed long-term effects, as the oxidation of the cellular lipids and damaged mitochondria. All these consequences of the oxidative stress generated by Mn exposure were not observed in the vinylic telluride-treated animals. These findings are probably due to the antioxidant activity of this organotellurium compound, which has been already reported *in vitro*. DPTVP can decrease the sodium nitroprusside (SNP), quinolinic acid and iron- induced TBARS formation in the brain of rodents at very low concentrations, indicating the high antioxidant potential of this compound (De Avila *et al.*, 2006; Avila *et al.*, 2008). In addition, the compound can protect against SNP- induced decrease in mitochondrial viability in slices of cortex, hippocampus and striatum, reinforcing the purpose about its antioxidant capacity (Avila *et al.*, 2008).

Another interesting finding was the recover provided by the DPTVP from the reduced [H^3] glutamate uptake in the animals exposed to Mn. It has been reported that Mn can cause such effect once it can accumulate in astrocytes and generates severe damage to them, impairing their glutamate uptake function (Pentschew *et al.*, 1963) with consequent overactivation of NMDA receptors, massive Ca^{2+} influx and ROS production (Choi, 1988). On the other hand, ROS can be generated by the damaged mitochondria, once Mn can accumulates in this organelle (Wedler *et al.*, 1989; Erikson *et al.*, 2004) and cause a dysfunction, as observed in this current investigation by the MTT assay. These species can oxidize cysteine residues of the GLASTs and decrease their function in the glutamate uptake process (Trotti *et al.*, 1998). It is not clear if the oxidative imbalance is the cause or the consequence of the reduced glutamate uptake and all other effects here demonstrated, however the treatment with the organotellurium compound showed the central role of oxidative stress in Mn's neurotoxicity and proves how helpful an antioxidant treatment may be against this disorder.

It has been massively reported that Mn`s overload may lead to manganism, a disorder characterized by a complex behavioral syndrome which includes motor deficits (Couper, 1837a). Motor control is mainly controlled by dopaminergic-rich areas, such as the striatum (Sloot e Gramsbergen, 1994). Nevertheless, striatum is a brain area with a high expression of the divalent metal transporter type 1 (DMT-1), which is responsible for Mn uptake across the blood brain barrier (Au *et al.*, 2008). This may explain the accumulation of the metal in this structure demonstrated here, as well as the higher susceptibility to Mn in comparison to cortex and hippocampus and the decrease in the motor control observed in the rotarod task in the Mn-treated animals. Nevertheless, our most inquiring finding was the decrease in the Mn levels in the striatum of the rats post-treated with DPTVP. There is no report in the literature regarding Te^{2+} transport into the CNS and how it could affect Mn^{2+} transport. Nevertheless, some studies with tellurite (Te^{3+}) have shown that it can be uptaked via a monocarboxylate transport system (Borghese *et al.*, 2008), similar to how Mn is also uptaked (Crossgrove *et al.*, 2003; Aschner *et al.*, 2007). Furthermore, Borsetti et al (Borsetti *et al.*, 2003) demonstrated that Te^{3+} can be also transported through a process which is pH dependent, suggesting that DMT-1 could be also responsible for this process, once it is a proton dependent transport. These results might be a clue, however, further studies are necessary to understand how Te competes with Mn to be transported and decrease its levels in a short period of treatment.

In summary, our results confirm the rising concern regarding Mn neurotoxicity and the important role that oxidative stress plays in this disorder at the cellular level, especially in the motor control areas as striatum. Moreover, we demonstrated the neuroprotective effects of the antioxidant compound DPTVP against Mn neurotoxicity. Further investigations concerning Te uptake into the CNS must be performed once our data suggest an interaction with Mn transport, which could be an interesting mechanism of defense against Mn poisoning. This line of research is very important in an attempt to find alternative treatments against Mn intoxication and its effects especially in the brain.

Figure Legends

Figure 1: Neurobehavioral evaluation in rats exposed to Mn for 4 months (137mg/Kg) and co-treated for 14 days with DPTVP (0.150µmol/Kg). (A) number of crossings in the open field; (B) number of rearing in the open field; (C) latency for the first fall in the rotarod. Each bar represents mean ± S.E.M (n=5). * indicates statistical difference from control group by one-way ANOVA, following by SNK post-hoc test ($p<0.05$).

Figure 2: Effects of the Mn exposure (137mg/Kg) and/or co-treatment with DPTVP (0.150µmol/Kg) on TBARS levels in cortex (A), hippocampus (B) and striatum (C) of treated rats. Data are expressed as nmols of MDA/mg of protein. Each bar represents mean ± S.E.M (n=5). * indicates statistical difference from control group by one-way ANOVA, following by SNK post-hoc test ($p<0.05$).

Figure 3: Mitochondrial viability in cortex (A), hippocampus (B) and striatum (C) of animals chronically exposed to Mn and co-treated for 14 days with DPTVP. Data are expressed as percentage of control. Each bar represents mean ± S.E.M (n=5). * indicates statistical difference from control group by one-way ANOVA, following by SNK post-hoc test ($p<0.05$).

Figure 4: [^{3}H]glutamate uptake in cortex (A), hippocampus (B) and striatum (C) of animals chronically exposed to Mn and co-treated for 14 days with DPTVP. Data are expressed as nmols of [^{3}H]glutamate/mg of protein/min. Each bar represents mean ± S.E.M (n=5). * indicates statistical difference from control group by one-way ANOVA, following by SNK post-hoc test ($p<0.05$).

Figure 5: Mn levels in rats`striatum after chronic Mn exposure and/or co-treatment with DPTVP. Data are expressed as µg of Mn/g of wet tissue. Each bar represents mean ± S.E.M (n=5). * indicates statistical difference from control group by one-way ANOVA, following by SNK post-hoc test ($p<0.05$).

Table 1: Body weight values (g) of control and Mn-treated rats after 4 months treatment and after DPTVP post-treatment

groups	BW0	BW 4 mo	groups	BW end
Control	227.9 ± 16.8	367.9 ± 13.9	CONTROL	387.8 ± 25.04
			DPTVP	375.6 ± 19.9
Mn	254.4 ± 12.7	379.3 ± 15.4	Mn	380.8 ± 22.44
			Mn+DPTVP	381.6 ± 20.8

Data are expressed as mean ± SEM for each group (n=5). BW0-body weight before Mn treatment; BW4mo- body weight after 4 months of Mn treatment; BWend- body weight after DPTVP co-treatment.

Table 2: Behavioral alterations in rats caused by 4 months of Mn treatment in the drinking water

	Open field		Rotarod task	
groups	n of crossings	n of rearings	time to the 1st fall (s)	n of falls
Control	11.6 ± 3.01	7.4 ± 2.18	110.8 ± 5.45	1.3 ± 0.26
Mn	8.7 ± 2.78	3.6 ± 0.81*	90.1 ± 10.56*	0.9 ± 0.45*

Data are expressed as mean ± SEM for each group (n=5). * indicates statistical difference from control group.

Table 3: SOD activity (UI/min) in cortex, hippocampus and striatum of treated rats.

groups	CORTEX	HIPPOCAMPUS	STRIATUM
Control	21.24 ± 4.92	30.39 ± 5.08	36.91 ± 4.54
DPTVP	28.96 ± 6.25	22.08 ± 4.44	30.75 ± 2.71
Mn	27.16 ± 6.79	25.28 ± 5.47	29.32 ± 2.84
Mn+ DPTVP	21.56 ± 4.30	24.62 ± 4.06	35.89 ± 5.19

Data are expressed as mean ± SEM for each group (n=5).

Figure 1

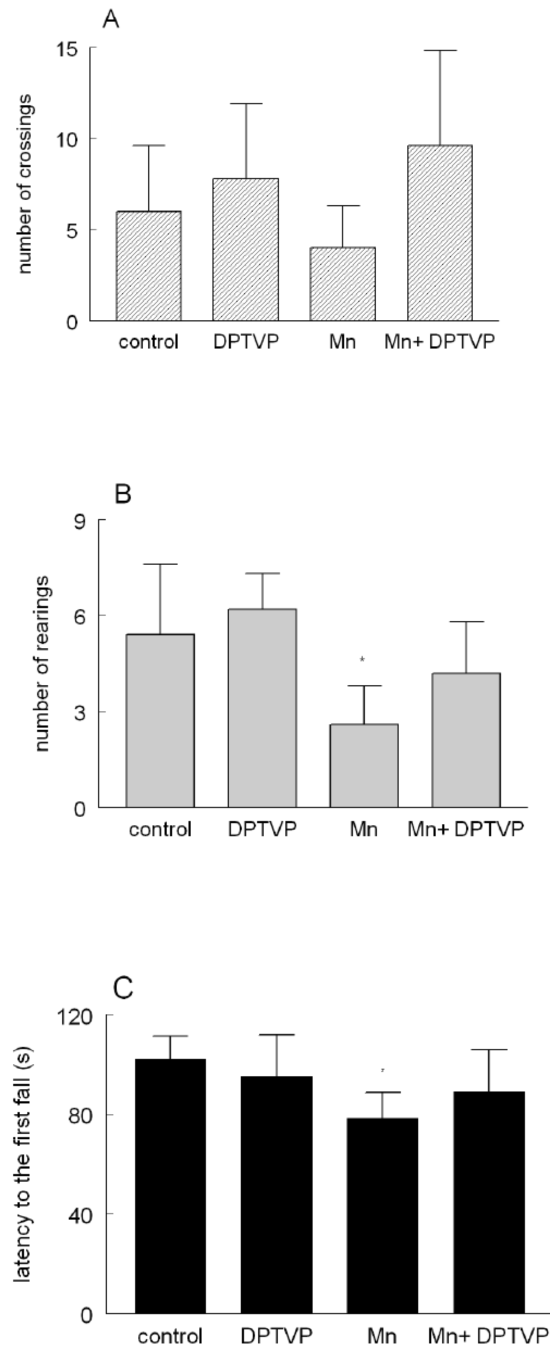
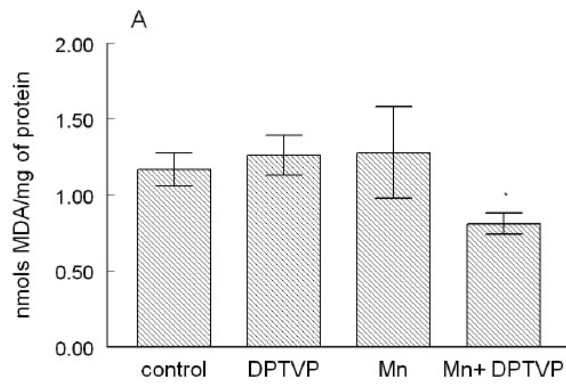
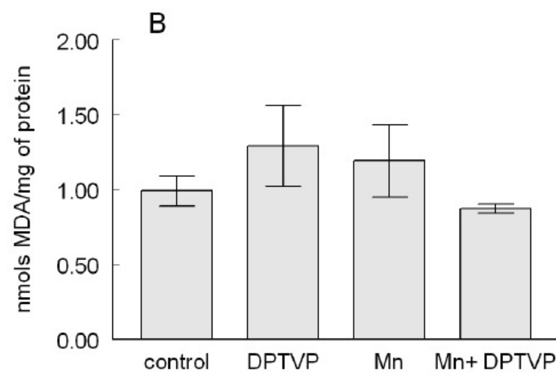


Figure 2





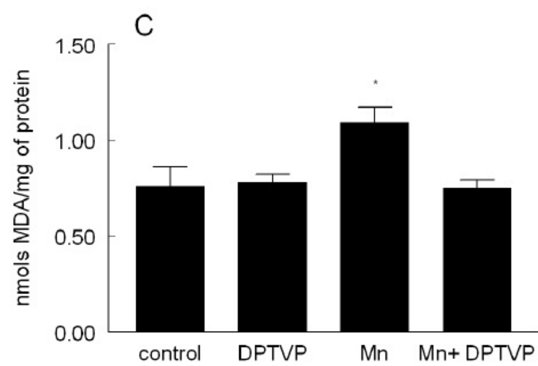


Figure 3

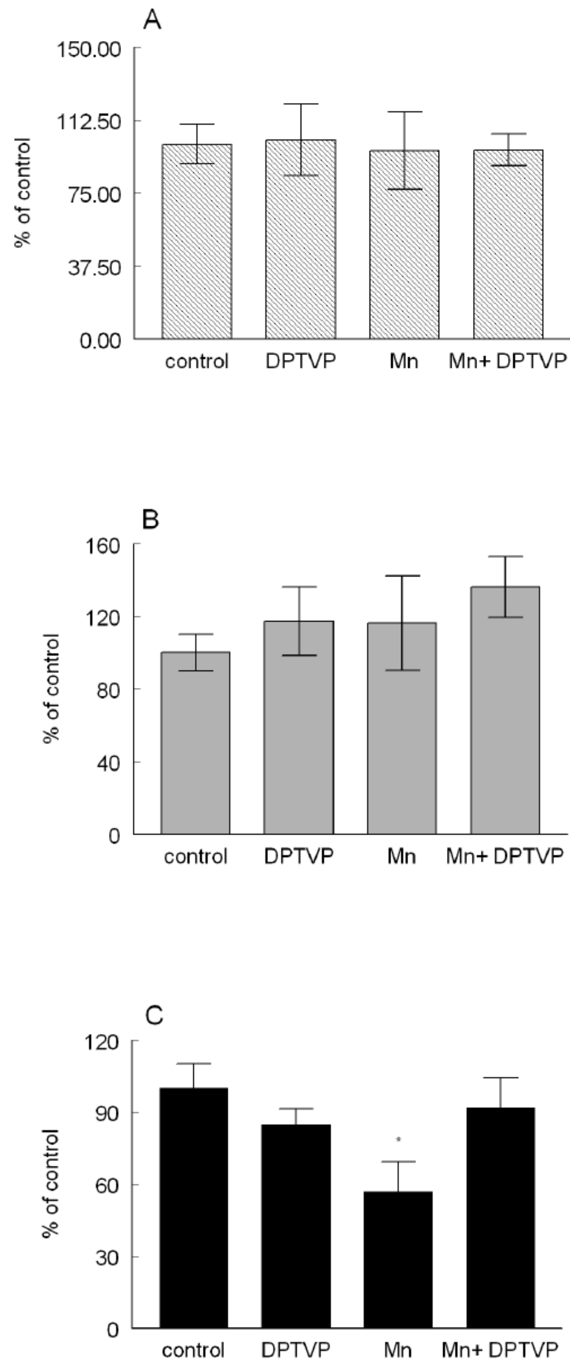


Figure 4

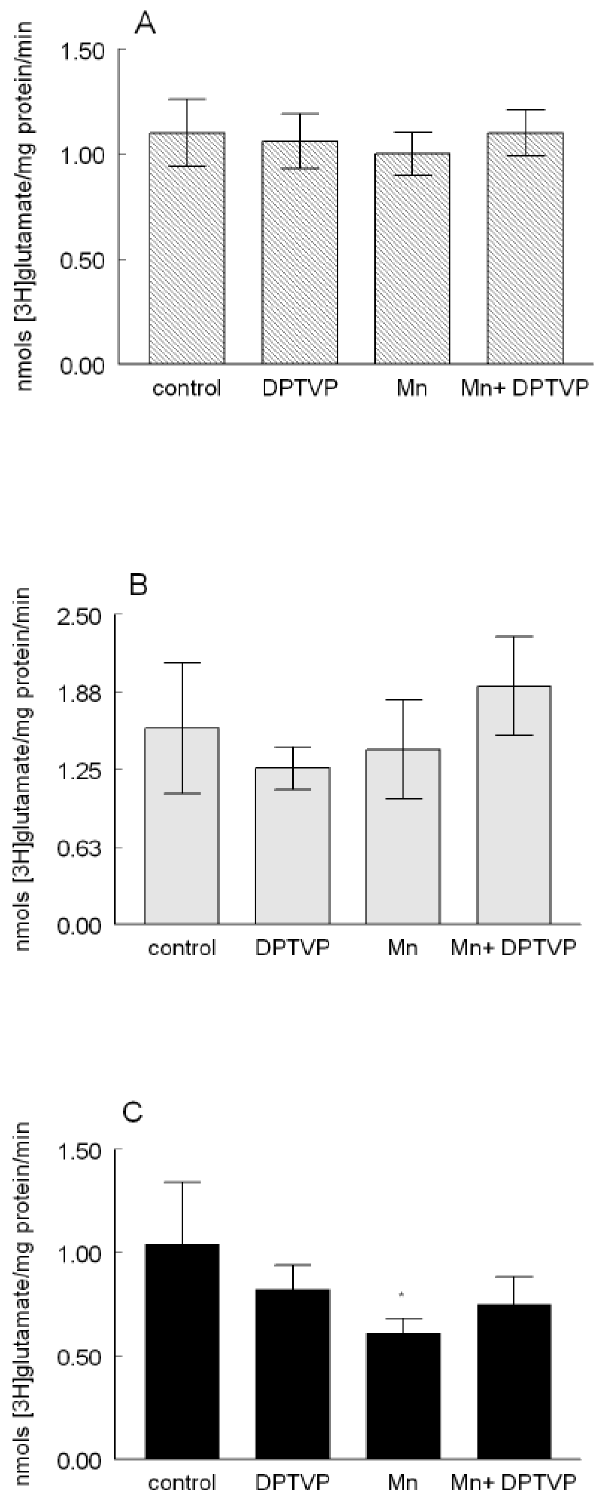
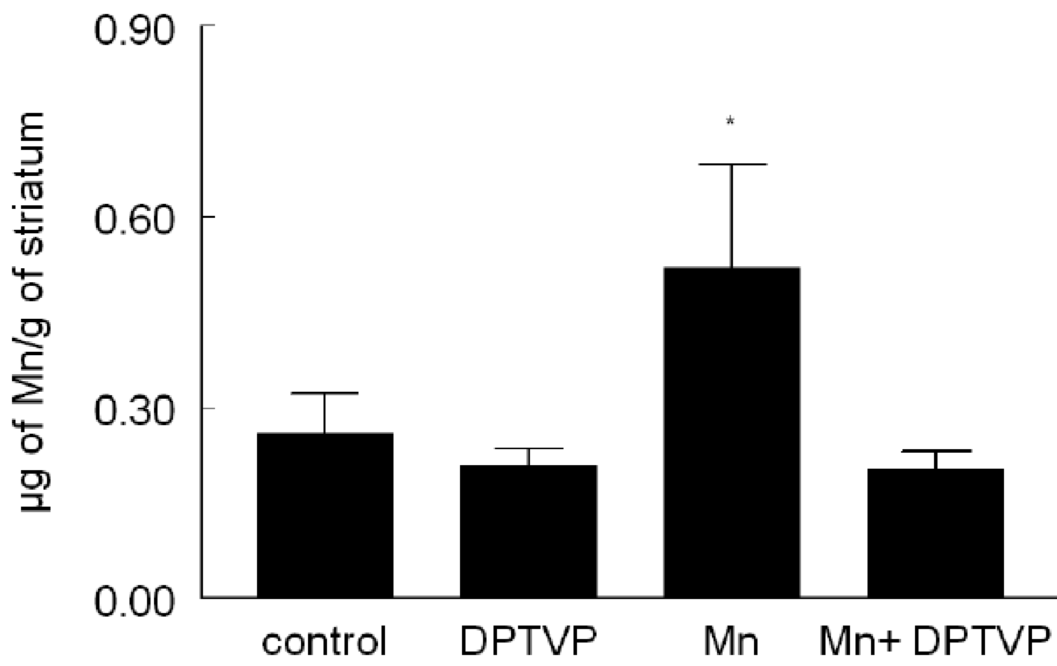


Figure 5



References

[1] A. Barbeau, Manganese and extrapyramidal disorders (a critical review and tribute to Dr. George C. Cotzias), Neurotoxicology 5(1) (1984) 13-35.
[2] J.D. Wang, C.C. Huang, Y.H. Hwang, J.R. Chiang, J.M. Lin, J.S. Chen, Manganese induced parkinsonism: an outbreak due to an unrepaired ventilation control system in a ferromanganese smelter, Br J Ind Med 46(12) (1989) 856-859.
[3] R.K. Hinderer, Toxicity studies of methylcyclopentadienyl manganese tricarbonyl (MMT), Am Ind Hyg Assoc J 40(2) (1979) 164-167.
[4] M.C. Newland, Animal models of manganese's neurotoxicity, Neurotoxicology 20(2-3) (1999) 415-432.
[5] H. Shinotoh, B.J. Snow, N.S. Chu, C.C. Huang, C.S. Lu, C. Lee, H. Takahashi, D.B. Calne, Presynaptic and postsynaptic striatal dopaminergic function in patients with manganese intoxication: a positron emission tomography study, Neurology 48(4) (1997) 1053-1056.
[6] W.N. Sloot, J.B. Gramsbergen, Axonal transport of manganese and its relevance to selective neurotoxicity in the rat basal ganglia, Brain Res 657(1-2) (1994) 124-132.
[7] E.P. Brouillet, L. Shinobu, U. McGarvey, F. Hochberg, M.F. Beal, Manganese injection into the rat striatum produces excitotoxic lesions by impairing energy metabolism, Exp Neurol 120(1) (1993) 89-94.
[8] M. Aschner, M. Gannon, H.K. Kimelberg, Manganese uptake and efflux in cultured rat astrocytes, J Neurochem 58(2) (1992) 730-735.
[9] A. Pentschew, F.F. Ebner, R.M. Kovatch, Experimental Manganese Encephalopathy in Monkeys. A Preliminary Report, J Neuropathol Exp Neurol 22 (1963) 488-499.
[10] J.B. Cavanagh, M.H. Kyu, Type II Alzheimer change experimentally produced in astrocytes in the rat, J Neurol Sci 12(1) (1971) 63-75.
[11] N.C. Danbolt, Glutamate uptake, Prog Neurobiol 65(1) (2001) 1-105.
[12] A.S. Hazell, M.D. Norenberg, Manganese decreases glutamate uptake in cultured astrocytes, Neurochem Res 22(12) (1997) 1443-1447.
[13] K. Erikson, M. Aschner, Manganese causes differential regulation of glutamate transporter (GLAST) taurine transporter and metallothionein in cultured rat astrocytes, Neurotoxicology 23(4-5) (2002) 595-602.
[14] D.W. Choi, Glutamate neurotoxicity and diseases of the nervous system, Neuron 1(8) (1988) 623-634.
[15] K.M. Erikson, A.W. Dobson, D.C. Dorman, M. Aschner, Manganese exposure and induced oxidative stress in the rat brain, Sci Total Environ 334-335 (2004) 409-416.
[16] M.A. Verity, Manganese neurotoxicity: a mechanistic hypothesis, Neurotoxicology 20(2-3) (1999) 489-497.
[17] F.C. Wedler, B.W. Ley, A.A. Grippo, Manganese(II) dynamics and distribution in glial cells cultured from chick cerebral cortex, Neurochem Res 14(11) (1989) 1129-1135.
[18] S. Zhang, J. Fu, Z. Zhou, In vitro effect of manganese chloride exposure on reactive oxygen species generation and respiratory chain complexes activities of mitochondria isolated from rat brain, Toxicol In Vitro 18(1) (2004) 71-77.
[19] C.E. Gavin, K.K. Gunter, T.E. Gunter, Mn2+ sequestration by mitochondria and inhibition of oxidative phosphorylation, Toxicol Appl Pharmacol 115(1) (1992) 1-5.

[20] K.V. Rao, M.D. Norenberg, Manganese induces the mitochondrial permeability transition in cultured astrocytes, J Biol Chem 279(31) (2004) 32333-32338.
[21] B. Halliwell, S. Chirico, Lipid peroxidation: its mechanism, measurement, and significance, Am J Clin Nutr 57(5 Suppl) (1993) 715S-724S; discussion 724S-725S.
[22] J.G. Greene, J.T. Greenamyre, Bioenergetics and glutamate excitotoxicity, Prog Neurobiol 48(6) (1996) 613-634.
[23] C.J. Chen, S.L. Liao, Oxidative stress involves in astrocytic alterations induced by manganese, Exp Neurol 175(1) (2002) 216-225.
[24] A.S. Hazell, L. Normandin, M.D. Norenberg, G. Kennedy, J.H. Yi, Alzheimer type II astrocytic changes following sub-acute exposure to manganese and its prevention by antioxidant treatment, Neurosci Lett 396(3) (2006) 167-171.
[25] L. Engman, J. Persson, K. Vessman, M. Ekstrom, M. Berglund, C.M. Andersson, Organotellurium compounds as efficient retarders of lipid peroxidation in methanol, Free Radic Biol Med 19(4) (1995) 441-452.
[26] A.C. Souza, C. Luchese, J.S. Santos Neto, C.W. Nogueira, Antioxidant effect of a novel class of telluroacetilene compounds: Studies in vitro and in vivo, Life Sci (2009).
[27] E. Wieslander, L. Engman, E. Svensjo, M. Erlansson, U. Johansson, M. Linden, C.M. Andersson, R. Brattsand, Antioxidative properties of organotellurium compounds in cell systems, Biochem Pharmacol 55(5) (1998) 573-584.
[28] B.P. Laden, T.D. Porter, Inhibition of human squalene monooxygenase by tellurium compounds: evidence of interaction with vicinal sulfhydryls, J Lipid Res 42(2) (2001) 235-240.
[29] C.W. Nogueira, G. Zeni, J.B. Rocha, Organoselenium and organotellurium compounds: toxicology and pharmacology, Chem Rev 104(12) (2004) 6255-6285.
[30] A.D. Toews, E.B. Roe, J.F. Goodrum, T.W. Bouldin, J. Weaver, N.D. Goines, P. Morell, Tellurium causes dose-dependent coordinate down-regulation of myelin gene expression, Brain Res Mol Brain Res 49(1-2) (1997) 113-119.
[31] V.C. Borges, L. Savegnago, S. Pinton, C.R. Jesse, D. Alves, C.W. Nogueira, Vinylic telluride derivatives as promising pharmacological compounds with low toxicity, J Appl Toxicol 28(7) (2008) 839-848.
[32] L. Savegnago, V.C. Borges, D. Alves, C.R. Jesse, J.B. Rocha, C.W. Nogueira, Evaluation of antioxidant activity and potential toxicity of 1-buthyltelurenyl-2-methylthioheptene, Life Sci 79(16) (2006) 1546-1552.
[33] D.S. Avila, P. Gubert, C.L. Dalla Corte, D. Alves, C.W. Nogueira, J.B. Rocha, F.A. Soares, A biochemical and toxicological study with diethyl 2-phenyl-2-tellurophenyl vinylphosphonate in a sub-chronic intraperitoneal treatment in mice, Life Sci 80(20) (2007) 1865-1872.
[34] D.S. Avila, P. Gubert, A. Palma, D. Colle, D. Alves, C.W. Nogueira, J.B. Rocha, F.A. Soares, An organotellurium compound with antioxidant activity against excitotoxic agents without neurotoxic effects in brain of rats, Brain Res Bull 76(1-2) (2008) 114-123.
[35] D.S. de Avila, M.C. Beque, V. Folmer, A.L. Braga, G. Zeni, C.W. Nogueira, F.A. Soares, J.B. Rocha, Diethyl 2-phenyl-2 tellurophenyl vinylphosphonate: an organotellurium compound with low toxicity, Toxicology 224(1-2) (2006) 100-107.
[36] D.S. Avila, A. Palma, D. Colle, R. Scolari, F. Manarin, A.F. Silveira, C.W. Nogueira, J.B. Rocha, F.A. Soares, Hepatoprotector activity of a vinylic telluride against acute exposure to acetaminophen., unpublished manuscript (2009).

[37] A.L. Braga, E.F. Alves, C.C. Silveira, L.H. Andrade, Stereoselective addition of sodium organyl chalcogenolates to alkynylphosphonates: synthesis of diethyl 2-(organyl)-2-(organochalcogenyl)vinylphosphonates, Tetrahedron Letters 41 (2000) 161-163.
[38] M.E. Burger, R. Fachinetto, G. Zeni, J.B. Rocha, Ebselen attenuates haloperidol-induced orofacial dyskinesia and oxidative stress in rat brain, Pharmacol Biochem Behav 81(3) (2005) 608-615.
[39] C. Rios, A. Santamaria, Quinolinic acid is a potent lipid peroxidant in rat brain homogenates, Neurochem Res 16(10) (1991) 1139-1143.
[40] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J Immunol Methods 65(1-2) (1983) 55-63.
[41] M.E. Frizzo, D.R. Lara, S. Prokopiuk Ade, C.R. Vargas, C.G. Salbego, M. Wajner, D.O. Souza, Guanosine enhances glutamate uptake in brain cortical slices at normal and excitotoxic conditions, Cell Mol Neurobiol 22(3) (2002) 353-363.
[42] H.P. Misra, I. Fridovich, The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase, J Biol Chem 247(10) (1972) 3170-3175.
[43] S.F. Ali, C.P. LeBel, S.C. Bondy, Reactive oxygen species formation as a biomarker of methylmercury and trimethyltin neurotoxicity, Neurotoxicology 13(3) (1992) 637-648.
[44] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J Biol Chem 193(1) (1951) 265-275.
[45] A. St-Pierre, L. Normandin, G. Carrier, G. Kennedy, R. Butterworth, J. Zayed, Bioaccumulation and locomotor effect of manganese dust in rats, Inhal Toxicol 13(7) (2001) 623-632.
[46] G. Cano, H. Suarez-Roca, E. Bonilla, Alterations of excitatory amino acid receptors in the brain of manganese-treated mice, Mol Chem Neuropathol 30(1-2) (1997) 41-52.
[47] D.C. Dorman, M.F. Struve, D. Vitarella, F.L. Byerly, J. Goetz, R. Miller, Neurotoxicity of manganese chloride in neonatal and adult CD rats following subchronic (21-day) high-dose oral exposure, J Appl Toxicol 20(3) (2000) 179-187.
[48] E.A. Malecki, Manganese toxicity is associated with mitochondrial dysfunction and DNA fragmentation in rat primary striatal neurons, Brain Res Bull 55(2) (2001) 225-228.
[49] D. Trotti, N.C. Danbolt, A. Volterra, Glutamate transporters are oxidant-vulnerable: a molecular link between oxidative and excitotoxic neurodegeneration?, Trends Pharmacol Sci 19(8) (1998) 328-334.
[50] J. Couper, On the effects of black oxide of manganese when inhaled into the lungs, British Annals of Medicine and Pharmacology 1 (1837) 41-42.
[51] C. Au, A. Benedetto, M. Aschner, Manganese transport in eukaryotes: the role of DMT1, Neurotoxicology 29(4) (2008) 569-576.
[52] R. Borghese, D. Marchetti, D. Zannoni, The highly toxic oxyanion tellurite (TeO (3) (2-)) enters the phototrophic bacterium Rhodobacter capsulatus via an as yet uncharacterized monocarboxylate transport system, Arch Microbiol 189(2) (2008) 93-100.
[53] M. Aschner, T.R. Guilarte, J.S. Schneider, W. Zheng, Manganese: recent advances in understanding its transport and neurotoxicity, Toxicol Appl Pharmacol 221(2) (2007) 131-147.
[54] J.S. Crossgrove, D.D. Allen, B.L. Bukaveckas, S.S. Rhineheimer, R.A. Yokel, Manganese distribution across the blood-brain barrier. I. Evidence for carrier-mediated influx of managanese citrate as well as manganese and manganese transferrin, Neurotoxicology 24(1) (2003) 3-13.

[55] F. Borsetti, A. Toninello, D. Zannoni, Tellurite uptake by cells of the facultative phototroph Rhodobacter capsulatus is a Delta pH-dependent process, FEBS Lett 554(3) (2003) 315-318.

IV.3. ATIVIDADE HEPATOPROTETORA DE UM TELURETO VINÍLICO CONTRA A EXPOSIÇÃO AGUDA DE ACETAMINOFENO

Manuscrito 2

HEPATOPROTECTOR ACTIVITY OF A VINYLIC TELLURIDE AGAINST ACUTE EXPOSURE TO ACETAMINOPHEN

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Hepatoprotector activity of a vinylic telluride against acute exposure to acetaminophen

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Abstract

Acetaminophen (APAP) hepatoxicity has been related with several cases of cirrhosis, hepatitis and suicides attempts. As oxidative stress plays a central role in the hepatic damage caused by APAP, antioxidants have been tested as alternative treatment against its toxicity. Here, we observed the hepatoprotective activity of the diethyl-2-phenyl-2-tellurophenyl vinylphosphonate (DPTVP), an organotellurium compound with low toxicity and high antioxidant potential. When the dose of 200mg/Kg of APAP was used, we observed that all used doses of DPTVP were able to restore the –SH levels that were depleted by APAP. Furthermore, the increase in TBARS levels and in the seric ALT activity, as well as the histopathological alterations caused by APAP were repaired to control levels by DPTVP (30, 50 and 100µmol/Kg). On the other hand, when the APAP dose used was 300mg/Kg, only the dose of 50µmol/Kg of DPTVP was able to restore the non proteic thiols levels and to repair the morphology of the intoxicated mice's livers. The other parameters altered by APAP were not improved by the post-treatment with the vinylic telluride. Our results demonstrate for the first time a pharmacological action of DPTVP as a hepatoprotector thanks probably to its antioxidant potential.

Keywords: tellurium, acetaminophen, oxidative stress, hepatoprotection, -SH levels, histophatological analysis.

1. Introduction

Acetaminophen (APAP) is a widespread and very effective drug used as analgesic and antipyretic. However, recent epidemiological studies have shown that the hospitalization rate due accidental or intentional APAP overdose is estimated to be over than 26,000 cases per year (Nourjah *et al.*, 2006), being considered as the major cause of liver failure (Larson *et al.*, 2005), hepatic transplant (Nourjah *et al.*, 2006) and a frequent reason of suicides attempts (Watson *et al.*, 2003). The mechanism of liver toxicity is not fully understood, however several studies in the literature demonstrate that the metabolization of this drug is responsible for the toxic effects. At therapeutic doses, APAP mainly undergoes glucuronidation and sulfation in the liver, which does not cause toxic effects (Prescott, 1980). Nevertheless, at over dosages, the citochrome P-450 system also metabolizes APAP, generating N-acetyl-*p*-benzoquinoneimine (NAPQI), a very toxic metabolite (Dahlin *et al.*, 1984), which is able to conjugate with GSH, depleting it from the hepatic stores (Mitchell, Jollow, Potter, Davis *et al.*, 1973) and to alkylate mitochondrial proteins (Jaeschke *et al.*, 2003; James *et al.*, 2003).

These happenings are the triggers of a cascade of events, starting with mitochondrial dysfunction and generation of reactive species (Cover *et al.*, 2005). The oxidative stress results in the opening of the mitochondrial membrane permeability transition pore, with rapid loss of ATP, inhibition of the calcium pumps and, finally, a massive Ca^{++} efflux to the citosol (Kass, 2006). This cellular collapse is concluded with hepatocytes death, which is characterized by the centrilobular necrosis with inflammatory cell proliferation, increase in plasmatic AST and ALT activities and GSH depletion (Nelson, 1990; Kon *et al.*, 2004; Oliveira *et al.*, 2005). Besides, APAP hepatotoxity also may cause haemostatic disorders such as the development of thrombosis and atherosclerosis, since coagulation and anticoagulation factors are produced in the liver (Hsu *et al.*, 2006).

In view of the fact that the imbalance of the oxidant/antioxidant system is the main consequence of the APAP- induced hepatotoxicity, many authors have been described the action of several antioxidants in different models of APAP intoxication in rodents. They demonstrated that different plant extracts (Kupeli *et al.*, 2006; Yen *et al.*, 2007) and

synthetic compounds (Srinivasan *et al.*, 2001) are able to diminish the hepatic biochemical and histological alterations caused by APAP. Of particular interest, organoselenium compounds, as ebselen and diphenyl diselenide were also evaluated, being the selenide ebselen a good agent against APAP- induced hepatotoxicity (Rocha *et al.*, 2005).

In this context, a variety of molecules containing selenium or tellurium, have been evaluated due to the pharmacological properties that they show in several models *in vitro* and *ex vivo*. Nevertheless, tellurium compounds are often very toxic to animals (Goodrum, 1998; Laden e Porter, 2001; Nogueira *et al.*, 2001), despite the higher antioxidant potential in comparison to organoselenides (Engman *et al.*, 1995; Wieslander *et al.*, 1998; Tiano *et al.*, 2000). On the other hand, we recently reported that a vinylic telluride, diethyl-2-phenyl-2-tellurophenyl vinylphosphonate possess an interesting antioxidant activity, being low toxic to mice exposed by intraperitoneal or subcutaneous routes for 12 days in doses reaching 500μmol/Kg of the compound (De Avila *et al.*, 2006; Avila *et al.*, 2007). Besides, the vinylic telluride is probably metabolized in the liver of rodents, once it was already demonstrated that the liver weight of treated mice increases, therefore the biochemical parameters of toxicity evaluated in this tissue were not affected, indicating that even at high concentrations in the liver, the compound do not cause any damage to the hepatocytes (Avila *et al.*, 2007). These characteristics become this vinylic telluride interesting in the research for useful antioxidant agents against the hepatic oxidative damage caused by APAP. Hence, in this study we evaluated the effects of the organotellurium compound against the biochemical and histological damage caused by a minimal dose of APAP able to cause hepatotoxicity and a high dose of APAP that causes severe damage to hepatic tissue.

2. Materials and Methods

2.1. Chemicals

Diethyl 2- phenyl-2-tellurophenyl vinylphosphonate was synthesized by the addition of alkynylphosphonates to a solution of sodium organyl tellurolate, prepared by the reduction of diorganyl ditellurides with sodium borohydride in ethanol at room temperature (Braga *et al.*, 2000b).

All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.2. Animals

Adult male albino mice (25-35g) from our own breeding colony were maintained in an air conditioned room (20–25°C) under natural lighting conditions with water and food (Guabi-RS, Brasil) *ad libitum.* All experiments were conducted in accordance with the Guiding Principles of the Animal Care and Wellness Committee of the Universidade Federal de Santa Maria.

2.3. Animals' Treatment

APAP was dissolved in warm saline and DPTVP in canola oil. A total of 50 animals were used in the experiments. Mice were divided into two different protocols:

1) 200mg/Kg of APAP p.o. (n=5 each group)
    - I) mice received vehicle (saline 0.9%, p.o.);
    - II) APAP (200mg/Kg, p.o.) + DPTVP vehicle (canola oil, i.p.) ;
    - III) APAP(200mg/Kg, p.o.) + DPTVP (30µmol/Kg, i.p.);
    - IV)APAP (200mg/Kg, p.o.) + DPTVP (50 µmol /Kg, i.p.);
    - V) APAP (200mg/Kg, p.o.) + DPTVP (100 µmol /Kg, i.p.).
2) 300mg/Kg of APAP p.o (n=5 each group);
    - I)mice received vehicle (saline 0.9%, p.o.);
    - II) APAP (300mg/Kg, p.o.) + DPTVP vehicle (canola oil, i.p.);

III) APAP(300mg/Kg, p.o.) + DPTVP (30 µmol /Kg, i.p.);

IV) APAP (300mg/Kg, p.o.) + DPTVP (50m µmol Kg, i.p.);

V) APAP (300mg/Kg, p.o.) + DPTVP (100 µmol /Kg, i.p.).

Animals received a single dose of APAP or vehicle, and after 30 minutes DPTVP or oil were administered. Twenty-four hours after DPTVP injection, mice were anesthetized with ether to heart blood puncture. Blood was collected in tubes containing heparine and plasma was obtained by centrifugation. Liver was removed and used for histological analysis or homogenized in Tris-HCl buffer (10mM, pH7.4) and then centrifuged (10 minutes, 2,000 xg, 4˚C) to obtain the low speed supernatant (S1) for the biochemical analysis. The APAP doses were chosen after dose responses curves that were done in our lab. The DPTVP doses used here do not have any toxic effects by the i.p route, as described in previous work (Avila *et al.*, 2007).

2.4. Biochemical Analysis:

2.4.1. TBARS formation

Lipid peroxidation in liver was assessed by the measurement of thiobarbituric reactive substances (Ohkawa *et al.*, 1979). TBARS were determined spectrophotometrically at 532nm after one hour of pre-incubation at 37°C and following by one hour of incubation with SDS 8.1%, Acetic acid/ HCl buffer and thiobarbituric acid 0.6% at 95ºC , using Malondialdehide as standard.

2.4.2. GSH content

Liver reduced glutathione content was estimated as non-proteic SH content using Ellman's reagent (DTNB 10mM) after deproteinization with TCA (10%), according with the standard methodology (Ellman, 1959).

2.4.3. δ- ALA- D activity

Liver δ-ALA-D activity was assayed according to the method of Sassa (1982) (Sassa, 1982), by measuring the rate of product (porphobilinogen, PBG) formation, except that 84 mM potassium phosphate buffer, pH 6.4, and 2.5 mM ALA were used. All

experiments were carried out after a 15 min preincubation of S1 with the medium, starting the reaction by adding the substrate, aminolevulinic acid. Incubation was carried out for 1 hour at 37°C. The reaction product was determined using modified Ehrlich's reagent at 555 nm, with a molar absorption coefficient of $6.1x10^4$ M^{-1} for the Ehrlich-porphobilinogen salt. The reactions rates were linear with respect to time of incubation and added protein for all the experimental conditions. Simultaneously, a set of tubes was assayed in the presence of 8 mM DTT to observe the possible reversion of the δ- ALA-D inhibition.

2.4.4. Superoxide dismutase activity

SOD was determined in S1 of liver according to the methodology of Misra and Fridowich (1972) (Misra e Fridovich, 1972). The adrenochrome production was measured spectrophotometrically at 480 nm. One unit of the enzyme was defined as the amount of enzyme required to inhibit the rate of adrenaline auto-oxidation by 50%.

2.4.5. Catalase activity

Catalase activity was assessed according to Aebi et al. (1984) (Aebi, 1984) in S1 of liver, measuring the rate of disappearance of H_2O_2 spectrophotometrically at 240nm. One unit of the enzyme is considered as the amount which decomposes 1µmol H_2O_2/min at pH 7.

2.4.6. Protein measurements

Liver proteins were determined using the colorimetric method of Lowry (1951) (Lowry *et al.*, 1951).

2.4.7. Plasmatic AST and ALT activities

Plasmatic aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were quantified as biochemical endpoints of hepatotoxicity using commercial kit (LABTEST, Diagnostica S.A., Minas Gerais, BR) and according to the method of Reitman and Frankel (1957) (Reitman e Frankel, 1957).

2.4.8. Histopathology

The liver of treated mice were carefully removed, washed in saline solution and then immersed in formalin 10%. For light microscopy examination, tissues were embedded in paraffin, sectionated in 5µm and stained with hematoxylin and eosin (n=3 per group).

Statistical Analysis

Statistical significance was assessed by one-way ANOVA, followed by Student Newman Keuls test for post hoc comparison. Results were considered statistically significant at values of $p<0.05$.

3. Results

3.1. Hepatotoxicity induced by APAP 200mg/Kg

The intoxication of mice with APAP at the dose of 200 mg/Kg lead to an impoverishment in liver GSH levels, observed by the reduction of non proteic–SH groups in treated mice ($p<0.05$, Figure 1A). In addition, a significant increase in TBARS levels was observed (Figure 1B), as well as inhibition of hepatic δ- ALA- D activity, which was not reverted by DTT ($p< 0.05$, Figure 1C). Also, there was a leakage of ALT activity in the plasma, reflecting the failure of the liver function due to APAP treatment ($p<0.05$, Table 1). On the other hand, the antioxidant biomarkers used as SOD and CAT activities were not altered by 200mg/Kg APAP administration (data not shown).

Interestingly, DPTVP was able to overturn the biochemical parameters in APAP-induced hepatoxicity. GSH depletion caused by APAP was restored by DPTVP at all tested doses ($p<0.05$, figure 1A). In accordance, the oxidative stress caused by GSH depletion was reverted by the organotellurium compound, as indicated by decreased MDA levels ($p<0.05$, figure 1B). Besides, ALT leakage was not observed from the dose of 30 µmol/Kg ($p<0.05$, Table1). The only parameter altered by APAP that was not restored by the compound was the activity of δ- ALA- D (figure 1C)

Figures 2A-E present the histology of mice's liver after different treatments. Light microscopic evaluation showed regular morphology of the liver parenchyma with well-

designed hepatic cells and sinusoids in control group (Figure 2A). The administration of the DPTVP alone did not cause any histological damage to liver (data not shown). The APAP treated group showed mild liver injury with accumulation of inflammatory cells, perinuclear vacuolization and swollen hepatocytes (Figure 2B). Post-reatment with DPTVP (30, 50 and 100µmol/Kg) evidenced the morphological improvement of liver after APAP exposure. Still, is possible to observe some inflammatory cells infiltration, vacuolization and binucleated hepatocytes, indicating regeneration (Figures 2C-E).

3.2. Hepatotoxicity by APAP 300mg/Kg

The liver injury caused by APAP was strongly enhanced by the raise of the dose administered to mice (Figures 3A-C, 4B, Table 2). This was detected by the lack of reversion effect of DPTVP at all tested doses in the biochemical parameters that were changed by APAP. Different from the intoxication with APAP 200mg/Kg, DPTVP was able to restore GSH levels only at 50 µmol/Kg ($p<0.05$, Figure 3A). The TBARS increment was not decreased by the treatment with the compound (Figure 3B). The injured hepatic function indicated by AST and ALT leakage to blood was not improved by the vinylic telluride at any of the tested doses (Table 2). Furthermore, the inhibition in hepatic δ- ALA-D activity was impaired by the treatment with DPTVP at 100µmol/Kg ($p<0.05$, Figure 3C). Once more, we did not observed alterations in hepatic SOD and CAT activities, even when the high dose was employed (data not shown).

In the histology of the APAP- treated livers, we observed severe liver injury with congestion of the sinusoidal space and high accumulation of inflammatory and red blood cells. Besides, swollen hepatocytes, perinuclear vacuolization and extensive necrosis in periportal region were seen (Figure 4B). The treatment with 30µmol/Kg of the vinylic phosphonate did not show any improvement. On the other hand, the slides from APAP + 50 or 100µmol/Kg of DPTVP treated livers illustrate the significant improvement of the morphological alterations caused by APAP alone. It is possible to note that there is still some polimorphonuclear infiltration, but the mild cellular and nuclear pleomorphism indicates the regeneration of the hepatic tissue (Figures 4C-E).

Discussion

In agreement with previous studies, we observed that acetaminophen administered orally to mice at mild doses caused biochemical and histological damage to hepatic tissue. Post-treatment with diethyl-2-phenyl-2-tellurophenyl vinylphosphonate, an organotellurium compound with low toxicity to mice, was able to restore biochemical parameters altered by APAP. In fact, the compound was very effective at a mild dose of APAP (200mg/Kg), however the severe biochemical damage caused by the highest dose of APAP used (300 mg/Kg) was not recovered by DPTVP doses.

Recently there has been much interesting in the role of the oxidative stress in the pathogenesis and progression of liver diseases, as well as in several models of liver damage caused by drugs, mainly in APAP- induced liver injury (Larson, 2007; Fontana, 2008). As the deliberated use of this drug is increasing, the research for new compounds which revert the oxidative stress in intoxicated hepatocytes without cause any damage to the liver and other organs is relevant, and several drugs have been tested, and some of them failed in this purpose (Rocha *et al.*, 2005; Olaleye e Rocha, 2008; Sabir e Rocha, 2008; Yalcin *et al.*, 2008). The vinylic telluride raised as an option to be tested as hepatoprotector once it is not toxic and do not cause any liver damage to mice, even when administered at high doses as 500 µmol/Kg for 12 days (De Avila *et al.*, 2006) besides the good antioxidant potential that has been reported by our group (Avila *et al.*, 2007; Avila *et al.*, 2008).

In our APAP exposure protocol, we observed that non-proteic- SH groups were affected by 200mg/Kg of APAP. It is known that the APAP metabolite NAPQI reacts rapidly with glutathione (which is 90% of the non-proteic SH content) (Nelson, 1990; Oz *et al.*, 2004), consequently exacerbating oxidative stress, as indicated by TBARS increased levels. Furthermore, we observed a rise in the seric ALT activity, which is strictly related to the liver damage caused by APAP. ALT is a cytoplasmatic enzyme that is released into circulation after structural damage to the hepatocytes, being used as a standard biomarker of liver damage (Sallie *et al.*, 1991). Probably due to its antioxidant activity, DPTVP, at all tested doses, was able to restore from APAP- induced SH

depletion. Moreover, we obtained decreased MDA levels in the liver of the animals treated with the vinylic telluride, which means that the oxidative stress was indeed restricted by the compound. Furthermore, the seric ALT activity increased by APAP was recovered to control levels by DPTVP. Confirming the biochemical evidences, the histopathological analysis showed the regeneration of the hepatic tissue when compared to APAP treated group, with remarkable signals of regeneration.

Based on current experiments that have been done in our lab, we believe that the mechanism by which the vinylic telluride was able to recover the liver from the oxidative stress generated by APAP is due to its scavenger activity against several reactive oxygen/nitrogen species (not published data). Therefore, differently from some described organochalcogens compounds, the vinylic telluride did not show GPX-like activity, which could explain the recover from the GSH depletion. Hence, the mechanism by which the DPTVP restored SH levels is still unclear.

On the other hand, the vinylic telluride could not restore from the δ-ALA- D inhibition caused by APAP treatment. δ-ALA D is an oxidative stress sensitive enzyme, being used as biomarker. In its active site there are two cisteinyl residues that can be easily oxidized by several agents (Farina *et al.*, 2003; Nogueira, Santos *et al.*, 2004; Perottoni *et al.*, 2004). Nevertheless, the inhibition of this enzyme by APAP in our experimental protocol is not related to the oxidation of the SH groups in the enzyme. We can infer this once the addition of DTT, a thiol donator, did not restore the δ-ALA D activity. These data are in accordance to Rocha et al., which observed the same pattern of inhibition in an APAP exposure in rats. The mechanism underlying the enzyme inhibition by APAP is not well understood, but probably involves oxidative stress at a level that cannot be restored by DTT.

As all tested doses of the compound recovered the mice`s livers after pre-treatment with 200mg/Kg of APAP, we decided to challenge the potential of the compound as hepatoprotector, using the 300mg/Kg dose. This dose caused severe damage and biochemical alterations in the hepatic tissue, as indicated by high activity of seric ALT and AST, increased TBARS levels, non-proteic SH depletion, δ- ALA D inhibition and remarkable histopathological alterations. We found out that only the dose

of 50µmol/Kg of the vinylic telluride restored SH levels and recovered the hepatocytes from the damage observed in the histological analysis. This finding suggests that normal levels of gluthatione are needed for the restoration of the hepatic morphology.

The liver damage caused by 300mg/Kg of APAP was so severe that even the DPTVP dose that restored SH levels was not able to recover ALT and AST seric activities and increased TBARS levels. Nevertheless, as we have seen some benefic effects in the histological analysis, we believe that a chronic post-treatment with low doses of the vinylic telluride could improve the hepatocytes from the liver damage induced by APAP.

It was reported that acute administration of high doses of APAP decreases SOD and CAT activities (Ghosh e Sil, 2007). Furthermore, some authors have found the same alterations just several days after APAP exposure (Olaleye e Rocha, 2008; Sabir e Rocha, 2008). In our experimental protocol we noted the absence of alterations in SOD and CAT activities. These findings are in agreement with a study from Cigremis et al., which demonstrated in rabbits that the mRNA expression levels of SOD and CAT were not altered by a unique and mild dose of APAP, which also caused TBARS increase and hepatic histological alterations (Cigremis *et al.*, 2009). They suggested that probably the activity of these enzymes do not have a critical role and may show dual functions in APAP-induced hepatotoxicty.

Overall, our study demonstrated for the first time the hepatoprotective activity of DPTVP in vivo in rodents. Previously, our lab established the antioxidant potential of this compound in several tissues, including the hepatic one against iron-induced lipoperoxidation (De Avila *et al.*, 2006), as well as its low toxicity to rodents. These results instigate us in the search for other benefic activities of the compound, but also demonstrate that additional studies regarding the elucidation of the hepatoprotector mechanism must be performed.

Figure Legends

Figure 1: Biochemical parameters evaluated in the liver of mice treated with APAP 200mg/Kg p.o. and post-treated after 30 min with DPTVP (0, 30, 50, 100µmol/Kg, i.p.). A) non-proteic –SH levels; B) TBARS levels; C) δ-ALA-D activity. Results are expressed as mean ± S.E.M (n=5). * indicates statistical difference from control group by one-way ANOVA, following by SNK post-hoc test ($p<0.05$)

Figure 2: Histopathological analysis of the liver of mice treated with APAP 200mg/Kg p.o. and post-treated after 30 min with DPTVP (0, 30, 50, 100µmol/Kg, i.p.). A) control B) APAP C) APAP+ DPTVP 30 D) APAP + DPTVP 50 E) APAP+ DPTVP 100. Arrows indicate APAP damage PV- perinuclear vacuolization; IC- inflammatory cells accumulation; SH- swollen hepatocytes; BC- binucleated cells. Magnification 200x

Figure 3: Biochemical parameters evaluated in the liver of mice treated with APAP 300mg/Kg p.o. and post-treated after 30 min with DPTVP (0, 30, 50, 100µmol/Kg, i.p.). A) non-proteic –SH levels; B) TBARS levels; C) δ-ALA-D activity. Results are expressed as mean ± S.E.M (n=5). * indicates statistical difference from control group by one-way ANOVA, following by SNK post-hoc test ($p<0.05$)

Figure 4: Histopathological analysis of the liver of mice treated with APAP 300mg/Kg p.o. and post-treated after 30 min with DPTVP (0, 30, 50, 100µmol/Kg, i.p.). A) control B) APAP C) APAP+ DPTVP 30 D) APAP + DPTVP 50 E) APAP+ DPTVP 100. Arrows indicate APAP damage N- necrosis; IC- inflammatory cells accumulation; CNP- cellular and nuclear pleomorphism; PV- perinuclear vacuolization. Magnification 200x

Figura 1A

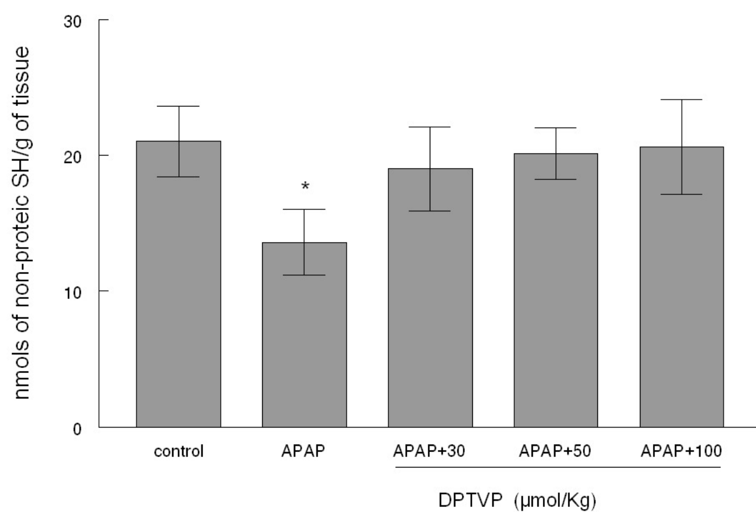


Figura 1B

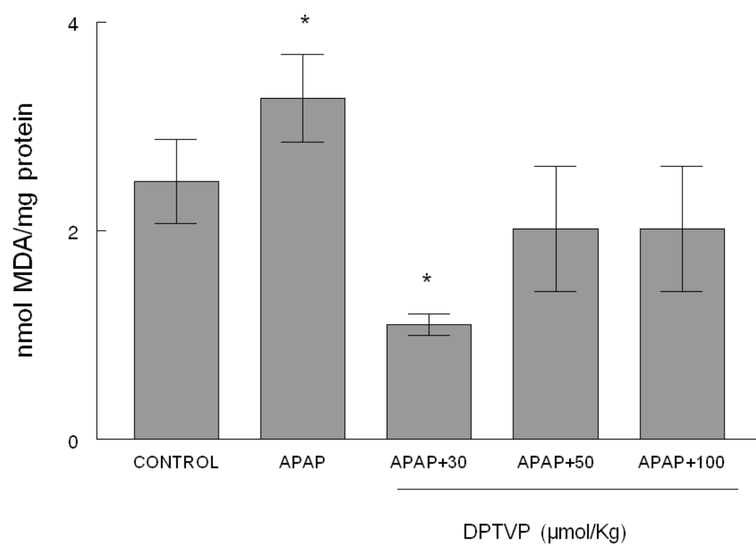


Figura 1C

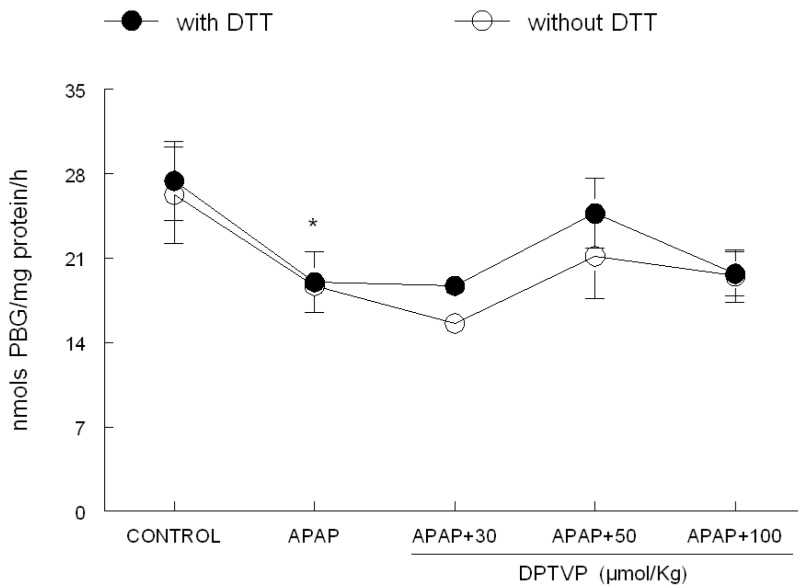


Table 1

Seric ALT and AST activities in mice after 24hs of the pre-treatment with APAP 200mg/Kg (p.o) followed by administration of 0,30, 50 or 100µmol/Kg of DPTVP (i.p).

Dose (µmol/Kg)	AST (U/mL)	ALT (U/mL)
Control	55 ± 0.5	34.1 ± 0.07
APAP	56.3 ± 0.5	45.5 ± 0.01*
APAP+DPTV (30)	48.1 ± 0.5	40 ± 0.3
APAP+DPTV (50)	59.2 ± 0.5	38.3 ± 0.3
APAP+DPTV (100)	55.4 ± 0.5	38.8 ± 0.3

Data are expressed as mean±S.E.M for five animals each group. * indicates statistical difference from control group ($p<0.05$).

Figura 2

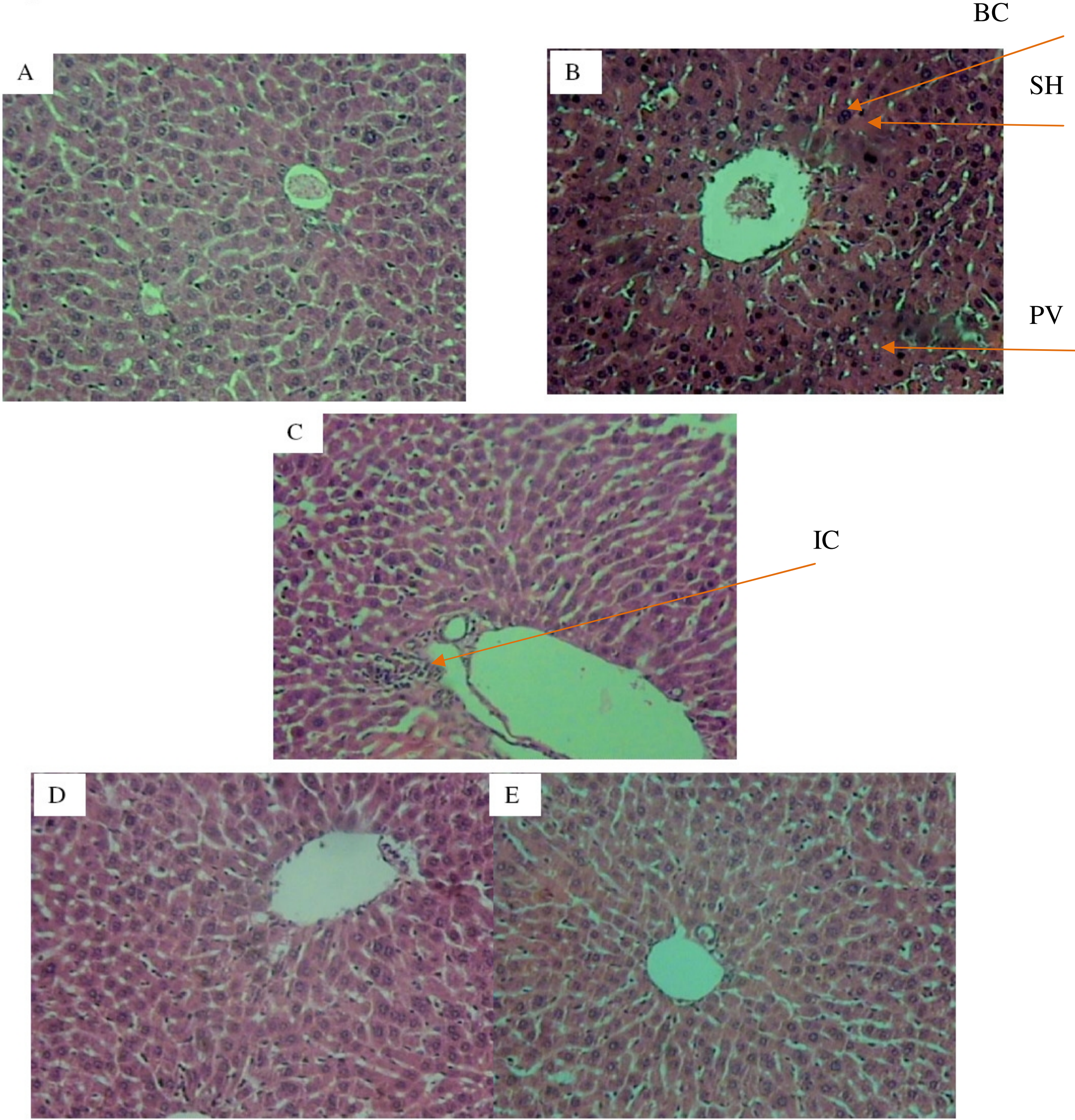


Figura 3A

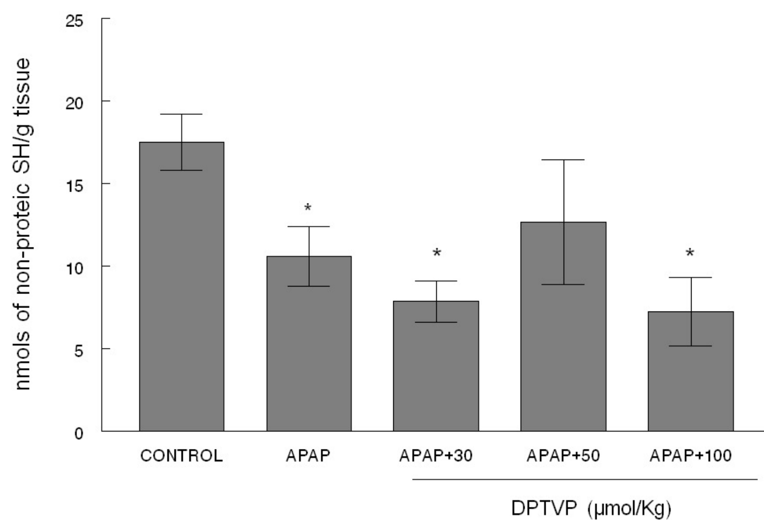


Figura 3B

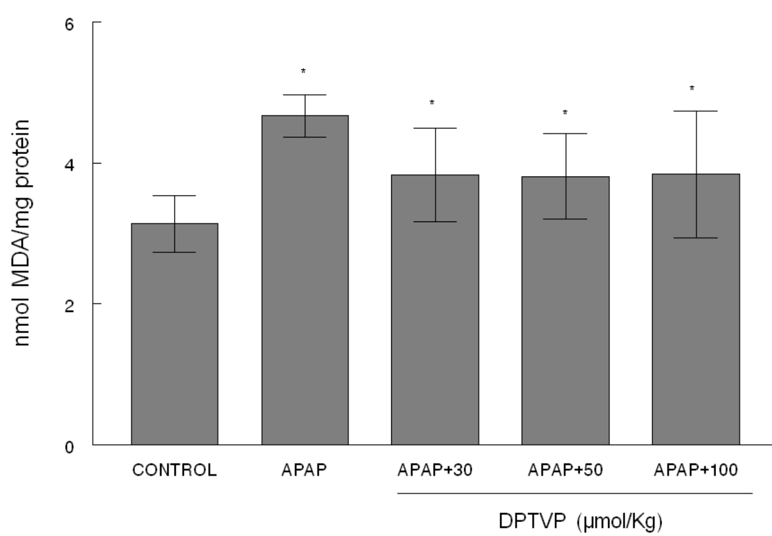


Figura 3C

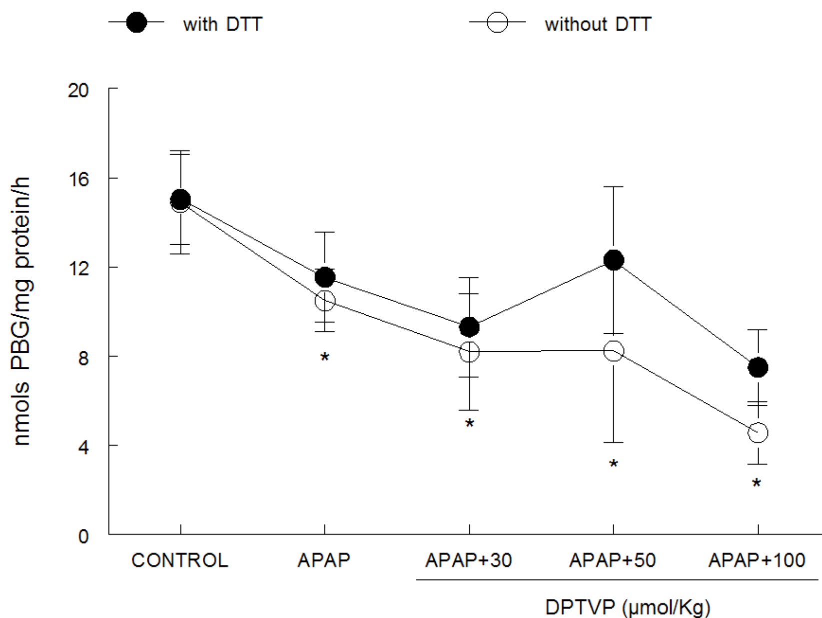


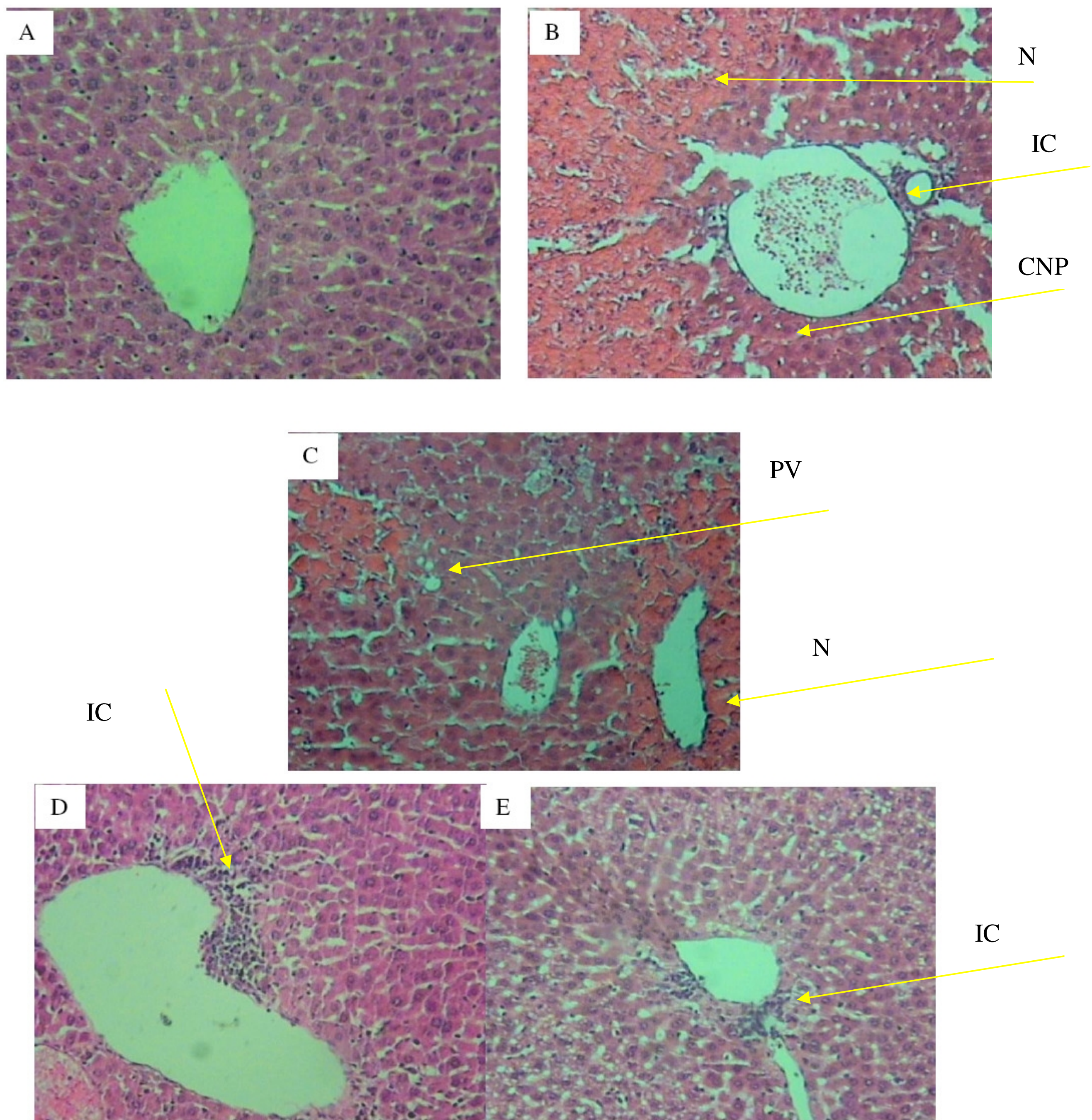
Table 2

Seric ALT and AST activities in mice after 24hs of the pre-treatment with APAP 300mg/Kg (p.o) followed by administration of 0,30, 50 or 100µmol/Kg of DPTVP (i.p).

Dose (µmol/Kg)	AST (U/mL)	ALT (U/mL)
Control	93 ± 8.8	31.4 ± 5.1
APAP	202.4 ± 14.7*	115.3 ± 12.1*
APAP+DPTV (30)	165.7 ± 5*	100.3 ± 17*
APAP+DPTV (50)	161.1 ± 10.1*	113.2 ± 30*
APAP+DPTV (100)	206.6 ± 25*	139.1 ± 1.7*

Data are expressed as mean±S.E.M for five animals each group. * indicates statistical difference from control group ($p<0.05$).

Figura 4



References

Aebi, H. (1984). Catalase in vitro. *Methods Enzymol* **105**, 121-126.

Avila, D. S., Gubert, P., Dalla Corte, C. L., Alves, D., Nogueira, C. W., Rocha, J. B., and Soares, F. A. (2007). A biochemical and toxicological study with diethyl 2-phenyl-2-tellurophenyl vinylphosphonate in a sub-chronic intraperitoneal treatment in mice. *Life Sci* **80**, 1865-1872.

Avila, D. S., Gubert, P., Palma, A., Colle, D., Alves, D., Nogueira, C. W., Rocha, J. B., and Soares, F. A. (2008). An organotellurium compound with antioxidant activity against excitotoxic agents without neurotoxic effects in brain of rats. *Brain Res Bull* **76**, 114-123.

Braga, A. L., Alves, E. F., Silveira, C. C., and Andrade, L. H. (2000). Stereoselective addition of sodium organyl chalcogenolates to alkynylphosphonates: synthesis of diethyl 2-(organyl)-2-(organochalcogenyl)vinylphosphonates. *Tetrahedron Letter* **41**, 161-163.

Cigremis, Y., Turel, H., Adiguzel, K., Akgoz, M., Kart, A., Karaman, M., and Ozen, H. (2008). The effects of acute acetaminophen toxicity on hepatic mRNA expression of SOD, CAT, GSH-Px, and levels of peroxynitrite, nitric oxide, reduced glutathione, and malondialdehyde in rabbit. *Mol Cell Biochem*.

Cover, C., Mansouri, A., Knight, T. R., Bajt, M. L., Lemasters, J. J., Pessayre, D., and Jaeschke, H. (2005). Peroxynitrite-induced mitochondrial and endonuclease-mediated nuclear DNA damage in acetaminophen hepatotoxicity. *J Pharmacol Exp Ther* **315**, 879-887.

Dahlin, D. C., Miwa, G. T., Lu, A. Y., and Nelson, S. D. (1984). N-acetyl-p-benzoquinone imine: a cytochrome P-450-mediated oxidation product of acetaminophen. *Proc Natl Acad Sci U S A* **81**, 1327-1331.

de Avila, D. S., Beque, M. C., Folmer, V., Braga, A. L., Zeni, G., Nogueira, C. W., Soares, F. A., and Rocha, J. B. (2006). Diethyl 2-phenyl-2 tellurophenyl vinylphosphonate: an organotellurium compound with low toxicity. *Toxicology* **224**, 100-107.

Ellman, G. L. (1959). Tissue sulfhydryl groups. *Arch Biochem Biophys* **82**, 70-77.

Engman, L., Persson, J., Vessman, K., Ekstrom, M., Berglund, M., and Andersson, C. M. (1995). Organotellurium compounds as efficient retarders of lipid peroxidation in methanol. *Free Radic Biol Med* **19**, 441-452.

Farina, M., Brandao, R., Lara, F. S., Soares, F. A., Souza, D. O., and Rocha, J. B. (2003). Mechanisms of the inhibitory effects of selenium and mercury on the activity of delta-aminolevulinate dehydratase from mouse liver, kidney and brain. *Toxicol Lett* **139**, 55-66.

Fontana, R. J. (2008). Acute liver failure due to drugs. *Semin Liver Dis* **28**, 175-187.

Ghosh, A., and Sil, P. C. (2007). Anti-oxidative effect of a protein from Cajanus indicus L against acetaminophen-induced hepato-nephro toxicity. *J Biochem Mol Biol* **40**, 1039-1049.

Goodrum, J. F. (1998). Role of organotellurium species in tellurium neuropathy. *Neurochem Res* **23**, 1313-1319.

Hsu, C. C., Lin, C. C., Liao, T. S., and Yin, M. C. (2006). Protective effect of s-allyl cysteine and s-propyl cysteine on acetaminophen-induced hepatotoxicity in mice. *Food Chem Toxicol* **44**, 393-397.

Jaeschke, H., Knight, T. R., and Bajt, M. L. (2003). The role of oxidant stress and reactive nitrogen species in acetaminophen hepatotoxicity. *Toxicol Lett* **144**, 279-288.

James, L. P., McCullough, S. S., Knight, T. R., Jaeschke, H., and Hinson, J. A. (2003). Acetaminophen toxicity in mice lacking NADPH oxidase activity: role of peroxynitrite formation and mitochondrial oxidant stress. *Free Radic Res* **37**, 1289-1297.
Kass, G. E. (2006). Mitochondrial involvement in drug-induced hepatic injury. *Chem Biol Interact* **163**, 145-159.
Kon, K., Kim, J. S., Jaeschke, H., and Lemasters, J. J. (2004). Mitochondrial permeability transition in acetaminophen-induced necrosis and apoptosis of cultured mouse hepatocytes. *Hepatology* **40**, 1170-1179.
Kupeli, E., Orhan, D. D., and Yesilada, E. (2006). Effect of Cistus laurifolius L. leaf extracts and flavonoids on acetaminophen-induced hepatotoxicity in mice. *J Ethnopharmacol* **103**, 455-460.
Laden, B. P., and Porter, T. D. (2001). Inhibition of human squalene monooxygenase by tellurium compounds: evidence of interaction with vicinal sulfhydryls. *J Lipid Res* **42**, 235-240.
Larson, A. M. (2007). Acetaminophen hepatotoxicity. *Clin Liver Dis* **11**, 525-548, vi.
Larson, A. M., Polson, J., Fontana, R. J., Davern, T. J., Lalani, E., Hynan, L. S., Reisch, J. S., Schiodt, F. V., Ostapowicz, G., Shakil, A. O., and Lee, W. M. (2005). Acetaminophen-induced acute liver failure: results of a United States multicenter, prospective study. *Hepatology* **42**, 1364-1372.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265-275.
Misra, H. P., and Fridovich, I. (1972). The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem* **247**, 3170-3175.
Mitchell, J. R., Jollow, D. J., Potter, W. Z., Davis, D. C., Gillette, J. R., and Brodie, B. B. (1973). Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. *J Pharmacol Exp Ther* **187**, 185-194.
Nelson, S. D. (1990). Molecular mechanisms of the hepatotoxicity caused by acetaminophen. *Semin Liver Dis* **10**, 267-278.
Nogueira, C. W., Rotta, L. N., Perry, M. L., Souza, D. O., and 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., Santos, F. W., Soares, F. A., and Rocha, J. B. (2004). 2,3-Dimercaptopropanol, 2,3-dimercaptopropane-1-sulfonic acid, and meso-2,3-dimercaptosuccinic acid inhibit delta-aminolevulinate dehydratase from human erythrocytes in vitro. *Environ Res* **94**, 254-261.
Nourjah, P., Ahmad, S. R., Karwoski, C., and Willy, M. (2006). Estimates of acetaminophen (Paracetomal)-associated overdoses in the United States. *Pharmacoepidemiol Drug Saf* **15**, 398-405.
Ohkawa, H., Ohishi, N., and Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* **95**, 351-358.
Olaleye, M. T., and Rocha, B. T. (2008). Acetaminophen-induced liver damage in mice: effects of some medicinal plants on the oxidative defense system. *Exp Toxicol Pathol* **59**, 319-327.
Oliveira, F. A., Chaves, M. H., Almeida, F. R., Lima, R. C., Jr., Silva, R. M., Maia, J. L., Brito, G. A., Santos, F. A., and Rao, V. S. (2005). Protective effect of alpha- and beta-amyrin, a triterpene mixture from Protium heptaphyllum (Aubl.) March. trunk wood resin, against acetaminophen-induced liver injury in mice. *J Ethnopharmacol* **98**, 103-108.

Oz, H. S., McClain, C. J., Nagasawa, H. T., Ray, M. B., de Villiers, W. J., and Chen, T. S. (2004). Diverse antioxidants protect against acetaminophen hepatotoxicity. *J Biochem Mol Toxicol* **18**, 361-368.

Perottoni, J., Lobato, L. P., Silveira, A., Rocha, J. B., and Emanuelli, T. (2004). Effects of mercury and selenite on delta-aminolevulinate dehydratase activity and on selected oxidative stress parameters in rats. *Environ Res* **95**, 166-173.

Prescott, L. F. (1980). Kinetics and metabolism of paracetamol and phenacetin. *Br J Clin Pharmacol* **10 Suppl 2**, 291S-298S.

Reitman, S., and Frankel, S. (1957). A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. *Am J Clin Pathol* **28**, 56-63.

Rocha, J. B. T., Gabriel, D., Zeni, G., Posser, T., Siqueira, L., Nogueira, C. W., and Folmer, V. (2005). Ebselen and diphenyl diselenide change biochemical hepatic responses to overdosage with paracetamol. *Environmental Toxicology and Pharmacology* **19**, 255-261.

Sabir, S. M., and Rocha, J. B. (2008). Antioxidant and hepatoprotective activity of aqueous extract of Solanum fastigiatum (false "Jurubeba") against paracetamol-induced liver damage in mice. *J Ethnopharmacol* **120**, 226-232.

Sallie, R., Tredger, J. M., and Williams, R. (1991). Drugs and the liver. Part 1: Testing liver function. *Biopharm Drug Dispos* **12**, 251-259.

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

Srinivasan, C., Williams, W. M., Ray, M. B., and Chen, T. S. (2001). Prevention of acetaminophen-induced liver toxicity by 2(R,S)-n-propylthiazolidine-4(R)-carboxylic acid in mice. *Biochem Pharmacol* **61**, 245-252.

Tiano, L., Fedeli, D., Santroni, A. M., Villarini, M., Engman, L., and Falcioni, G. (2000). Effect of three diaryl tellurides, and an organoselenium compound in trout erythrocytes exposed to oxidative stress in vitro. *Mutat Res* **464**, 269-277.

Watson, W. A., Litovitz, T. L., Rodgers, G. C., Jr., Klein-Schwartz, W., Youniss, J., Rose, S. R., Borys, D., and May, M. E. (2003). 2002 annual report of the American Association of Poison Control Centers Toxic Exposure Surveillance System. *Am J Emerg Med* **21**, 353-421.

Wieslander, E., Engman, L., Svensjo, E., Erlansson, M., Johansson, U., Linden, M., Andersson, C. M., and Brattsand, R. (1998). Antioxidative properties of organotellurium compounds in cell systems. *Biochem Pharmacol* **55**, 573-584.

Yalcin, S., Bilgili, A., Onbasilar, I., Eraslan, G., and Ozdemir, M. (2008). Synergistic action of sodium selenite and N-acetylcysteine in acetaminophen-induced liver damage. *Hum Exp Toxicol* **27**, 425-429.

Yen, F. L., Wu, T. H., Lin, L. T., and Lin, C. C. (2007). Hepatoprotective and antioxidant effects of Cuscuta chinensis against acetaminophen-induced hepatotoxicity in rats. *J Ethnopharmacol* **111**, 123-128.

IV.4. ESTUDOS SOBRE O MECANISMO ANTIOXIDANTE DO DIETIL-2-FENIL-2-TELUROFENIL VINILFOSFONATO

Manuscrito 3

STUDIES ON THE ANTIOXIDANT MECHANISM OF DIETHYL-2-PHENYL-2-TELLUROPHENYL VINYLPHOSPHONATE

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Em fase de redação

Studies on the antioxidant mechanism of diethyl-2-phenyl-2-tellurophenyl vinylphosphonate

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Abstract

Diethyl-2-phenyl-2-tellurophenyl vinylphosphonate (DPTVP) has been described as a very low toxic compound to rodents with hepato and neuroprotective activity thanks to its antioxidant activity. However, the mechanisms which underlie these activities are no clear. The objective of this study was to clarify which reactive oxygen and nitrogen species can be scavenged by DPTVP. We observed that the vinylic telluride has the ability to scavenge H_2O_2, OH•, NO• and $ONOO^-$. Furthermore, we observed that the potential of the antioxidant activity is dependent on the presence/absence of tissue, once SNP- induced protein carbonylation was decreased from only 2 µM, whereas the scavenging abilities were observed from 50 µM. We suggest that the ROS/RNS scavenger activity depicted by DPTVP is dependent on the phosphonate group present in its chemical structure, which provides the formation of the telluroxide form, as previously described by other authors with other organotellurium compounds. Nevertheless, additional studies are necessary to understand the metabolites that can be generated from DPTVP and their role in its antioxidant activity.

Keywords: tellurium, antioxidant, scavenger, reactive oxygen/nitrogen species, telluroxide.

1. Introduction

Reactive oxygen and nitrogen species (ROS/RNS) are molecules produced under normal conditions in eukaryotic cells, playing an important role in processes such as signaling pathways and cellular defense (Droge, 2002). Nevertheless, these molecules are very reactive, once they contain one or more unpaired electrons, being capable of independent existence, so called free radicals (Southorn e Powis, 1988). Diverse ROS and RNS are produced in large amounts during several diseases and infections, causing an imbalance between pro-oxidants and antioxidant within the cell, a condition well-known as oxidative stress (Halliwell e Gutterridge, 2003). Common causes of oxidative stress include toxemias, infections, hypoxia-ischemia, hyperglycemia, xenobiotics (drug metabolism), hyperlipidemias, hyperproteinemias, cancer, inflammation, phagocytic and immune reactions, and elevated metabolic rates (Mandelker, 2008).

In order to eliminate and defend against these highly reactive species, the body is provided with antioxidant enzymes known as primary antioxidants. Catalase, glutathione peroxidase and superoxide dismutase (SOD) are responsible for neutralize such species (Halliwell e Gutterridge, 2003). Furthermore, the cells are provided with non-enzymatic antioxidants proceeding from the diet, such as vitamin C and E, zinc, coenzyme Q, α-lipoic acid selenium (Mandelker, 2008). As during some diseases these antioxidants are not enough, several natural and synthetic antioxidants substances have been tested in order to find better therapeutically options, with high antioxidant potential and low toxicity (Wagner *et al.*, 2006; Pereira *et al.*, 2008; Puntel *et al.*, 2009; Sudati *et al.*, 2009).

In this context, several organotellurium compounds have shown antioxidant properties in different models in vitro and ex vivo (Andersson *et al.*, 1994; Engman *et al.*, 1997; Briviba *et al.*, 1998; Ren *et al.*, 2001). Nevertheless, some of them are very toxic, especially to the central nervous system (Toews *et al.*, 1997; Goodrum, 1998; Laden e Porter, 2001; Schiar *et al.*, 2009). On the other hand, vinylic tellurides have been reported as potent antioxidants with low toxicity to rodents (Savegnago *et al.*, 2006; Borges *et al.*, 2008; Souza *et al.*, 2009a) Diethyl-2-phenyl-2-tellurophenyl

vinylphosphonate (DPTVP) belongs to this class of tellurides with low toxicity, showing antioxidant activity, hepato and neuroprotective properties (De Avila *et al.*, 2006; Avila *et al.*, 2007; Avila *et al.*, 2008; Avila, D.S. *et al.*, 2009). However, the exact mechanism by which the compound exerts its activities is poorly understood.

Thus, to elucidate these questions we aimed to investigate the scavenger ability of DPTVP against different form of reactive species such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH•), nitric oxide radical (NO•), and 1,1-diphenyl-2-picrylhydrazyl(DPPH•). Furthermore, we would like to determine whether the compound could protect against protein oxidation, once this is a more reliable marker of oxidative stress.

2. Materials and Methods

2.1. Chemicals

Diethyl 2- phenyl-2-tellurophenyl vinylphosphonate (Figure 1) was synthesized by the addition of alkynylphosphonates to a solution of sodium organyl tellurolate, prepared by the reduction of diorganyl ditellurides with sodium borohydride in ethanol at room temperature (Braga *et al.*, 2000b).

All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.2. Animals

Adult male albino mice (25-35g) and Wistar rats (250-300g) from our own breeding colony were maintained in an air conditioned room (20–25°C) under natural lighting conditions with water and food (Guabi-RS, Brasil) *ad libitum.* All experiments were conducted in accordance with the Guiding Principles of the Animal Care and Wellness Committee of the Universidade Federal de Santa Maria.

2.3. In vitro tests without tissue

2.3.1.Chemioluminescence assay

The scavenging of hydrogen peroxide (H_2O_2) was developed by chemiluminescence assay according to (Singh *et al.*, 1988). Briefly, a range of DPTVP concentrations were incubated with 10 µM luminol. The reaction was started by the addition of H_2O_2 (50 µM). Chemiluminescence values were measured in counts per minutes (cpm). The DPTVP effect is expressed as % of control (without DPTVP).

2.3.2.Deoxyribose degradation

The deoxyribose degradation assay was performed according to (Puntel *et al.*, 2005). Briefly, the reaction medium was prepared containing the following reagents at the final concentrations indicated: DPTVP (concentrations indicated in the figures), deoxyribose 3mM, ethanol 5%, potassium phosphate buffer 0.05mM, pH 7.4, $FeSO_4$ 50 µM and H_2O_2 500 µM. Solutions of $FeSO_4$ and H_2O_2 were made prior to use. Reaction mixtures were incubated at 37 ◦C for 30 min and stopped by the addition of 0.8 mL of trichloroacetic acid (TCA) 2.8% followed by the addition of 0.4mL of thiobarbituric acid (TBA) 0.6%. Next, the medium was incubated at 100 ◦C for 20 min and the absorbance was recorded at 532 nm (Gutteridge, 1981; Halliwell e Gutteridge, 1981). Standard curves of malondialdehyde (MDA) were made in each experiment in order to determine the MDA generated by the deoxyribose degradation. The values are expressed as percentage of control values (without DPTVP).

2.3.3.DPPH• radical scavenging activity

The measurement of the scavenging activity of the DPTVP against the radical DPPH• was performed in accordance with (Choi *et al.*, 2002). Briefly, 85 µM DPPH• was added to a medium containing different DPTVP concentrations. The medium was incubated for

30min at room temperature. The decrease in absorbance measured at 518 nm depicted the scavenging activity of the DPTVP against DPPH•. The values are expressed in percentage of inhibition of DPPH• absorbance in relation to the control values without the DPTVP.

2.3.4.NO• scavenger activity

The scavenging of NO• was assessed by incubating sodium nitroprusside (SNP) (5 mM, in PBS) with different DPTVP concentrations at 25 ◦C. After 120 min, 0.5 mL of incubation solution was sampled and mixed with 0.5mL of Griess reagent (Green *et al.*, 1982). The absorbance was measured at 550 nm. The amount of nitrite was calculated using different concentrations of sodium nitrite. A curve of sodium nitrite, constructed in the presence of the DPTVP in order to verify its interaction with nitrite depicted no interference of the DPTVP in the color development after Griess reagent addition. The values were compared to control to determine the percentage of inhibition of nitrite reaction with Griess reagent depicted by the telluride as an index of its NO• scavenging activity (Marcocci *et al.*, 1994).

2.4. In vitro test with tissue

Animals were anesthetized with ether and decapitated. The liver, kidney and brain were removed and homogenized (1:20) in 10mM Tris buffer (pH 7.4) for protein carbonylation content determination.

2.4.1. Protein Carbonylation content

Liver, kidney and brain homogenates were pre- incubated with SNP (5 µM) and with DPTVP (0, 1, 4, 10 and 40 µM) for 12 hs. After, samples were used for the measurement of protein carbonyls content,-according to a modified method by (Levine *et al.*, 1990). Briefly, 1mL aliquots were mixed with 0.2 mL of 2,4 dinitrophenylhydrazine (10 mM DNPH) or 0.2 mL HCl (2M- blank). After 1h incubation at room temperature in a dark

environment, 0.5 mL of denaturing buffer (150 mM sodium phosphate buffer, pH 6.8, containing 3% of SDS), 1.5mL of ethanol and 1.5 mL of heptane were added and mixed for 40s by vortex agitation, and subsequently centrifuged for 15 min at 2,000xg. Next, the isolated protein in the interface ethanol:heptane was washed twice with ethyl acetate/ethanol 1:1 (v/v) and suspended in 1 mL of denaturing buffer. Each sample was measured at 370 nm in a Hitachi U-2001 spectrophotometer and compared to a corresponding blank sample. Total carbonylation was calculated using a molar extinction coefficient of 22,000M^{-1} cm^{-1}.

2.4.3. Protein content determination

Aliquots from the homogenates were separated to protein measurements that were assessed according to (Lowry *et al.*, 1951).

2.5. Statistical Analysis

Statistical significance was assessed by one-way ANOVA, followed by Duncan test for post hoc comparison. Results were considered statistically significant when $p<0.05$.

3. Results

3.1 in vitro assays without tissue

Figure 2 shows the scavenger activity of H_2O_2 by DPTVP using the chemioluminescence assay. It is possible to observe a biphasic curve, once the scavenging effect was observed at 1 µM and then only from 50 µM ($p<0.05$). In order to evaluate whether the compound could interfere with the Fenton reaction, we used the deoxyribose degradation assay (Figure 3). We observed again the H_2O_2- scavenger activity by DPTVP (from 50µM) when only this ROS was added to the medium ($p<0.05$). Furthermore, in the Fenton`s reaction conditions (Fe^{2+} + H_2O_2), the deoxyribose degradation was higher than in the H_2O_2 condition, due to the formation of OH•. DPTVP was able to decrease deoxyribose degradation caused by H_2O_2 and by Fenton reaction at the concentration of 100 µM.

To confirm the OH•- scavenging ability by DPTVP, we used the DPPH• assay. We observed an inhibition of the DPPH• reduction, indicating a scavenging activity from 50µM ($p<0.05$, Figure 4). Furthermore, the vinylic telluride also was able to scavenge NO• from 50 µM, once it was able to ($p<0.05$, Figure 5).

3.1 in vitro assays with tissue

Figures 6A-C illustrate the antioxidant effect of DPTVP against SNP-induced protein carbonylation in liver, kidney and brain, respectively. In the liver (Figure 6A), DPTVP showed a per se effect from 4µM, protecting against the carbonylation under normal conditions ($p<0.05$). Furthermore, SNP caused a significant increase in the protein carbonyl content, which was reverted by DPTVP from the concentration of 4µM ($p<0.05$). In the kidney (Figure 6B), there was no per se effect by DPTVP, nevertheless the carbonyl content which was increased by SNP returned to the control levels from the concentration of 1µM ($p<0.05$). In the brain (Figure 6C), DPTVP decreased the SNP-induced protein oxidation from 4µM, without any per se effect ($p<0.05$).

4. Discussion

This current investigation depicted interesting H_2O_2, NO•, $ONOO^-$ and OH• scavenging activity by DPTVP, suggesting that the antioxidant property of this compound could be attributable to its scavenger ability. Differentially from selenium (Se), which belongs to the same chemical family, tellurium is not an essential element to the body. Se is required for the function of the glutathione-peroxidase (GPX), and several organoselenium compounds have antioxidant activity due to their ability to mimic this enzyme (Nogueira, Zeni *et al.*, 2004). As Te and Se share chemical characteristics, such as reactivity, it is believed that Te could act as Se and exert the same functions (Nogueira, Zeni *et al.*, 2004). In fact, organotellurium compounds are described as better antioxidants and chemoprotectors than their Se analogues (Engman *et al.*, 1994; Rooseboom *et al.*, 2002) Accordingly to Andersson et al. (1994), the potency of the compounds, particularly in comparison with organoselenium analogues, suggest that

they may produce regenerable products during the inhibition of the peroxidation process (Andersson *et al.*, 1994).

Here, we evaluated the ability of the DPTVP to scavenge H_2O_2 using a chemioluminescence method, once luminol can be oxidized by H_2O_2 and generates a luminescent product, the aminophtalate (Giokas *et al.*, 2007). H_2O_2 is relatively the more stable ROS, once does not have an unpaired electron, however is one of the major sources of intracellular reactive species. H_2O_2 is generated intracellularly by several processes, such as by xanthine and urate oxidases and as a final product of the dismutation of the radical anion superoxide ($O_2{\bullet}^-$) (Mandelker, 2008). We observed that DPTVP decreased the luminescence at 1µM and then only at 50 and 100 µM. The scavenging activity provided by DPTVP against H_2O_2 is very important, considering that this ROS plays a central role in the generation of other ROS in several diseases (Halliwell e Gutterridge, 2003).

H_2O_2 in excess can degrade certain haem proteins such as myoglobin and hemoglobin, releasing iron (Fe^{2+}) (Halliwell e Gutterridge, 2003). This metal can react with H_2O_2 via the well known Fenton reaction, generating OH• radical, the most reactive specie (Halliwell e Gutteridge, 1984). To mimic this reaction, we used the deoxyribose degradation assay (Gutteridge, 1987), which uses a system containing Fe^{2+} and H_2O_2 to generate OH•. These radicals attack the 2-deoxy-D-ribose, degrading it into a series of fragments, some or all of them reactive to thiobarbituric acid. We observed that DPTVP was able to decrease the colorimetric reaction, verifying the ability of the compound in interacts with the Fenton reaction.

In order to confirm whether the interaction of the DPTVP with the Fenton reaction was due to its OH• scavenging activity, we performed the DPPH assay. The DPPH• radical is an one electron radical very similar to OH•, being used to evaluate the OH• scavenging (Choi *et al.*, 2002). The concentration which was able to scavenge the DPPH radical was lower that the observed in the deoxyribose assay, probably because of the absence of the agents which keep generating the ROS as in the deoxyribose degradation assay`s medium.

We attribute the scavenger activity depicted by DPTVP against ROS to its chemical structure. DPTVP has a phosphonate group which is an electron-withdrawing group. This group causes a decrease in the electronic density in the Te atom, which becomes Te^{+}, an eletrophilic specie. Considering that the ROS have an unpaired electron, we purpose that the Te^{+} can accept this electron, becoming telluroxide (Te IV), similar to which is described for several organotellurium compounds, with symmetrical structure or not (Andersson *et al.*, 1994; Engman *et al.*, 1994; Briviba *et al.*, 1998; Braga *et al.*, 2009). This mechanism can explain the scavenging of H_2O_2 and oxygen singlet, including in some RNS such as NO•.

Indeed, according to our results the DPTVP has a NO• scavenger activity. NO• is a very important signaling molecule, however is harmful in the presence of $O_2\bullet^-$. Such effect can be attributable to the formation of the metabolite peroxynitrite ($ONOO^-$) (Halliwell e Gutterridge, 2003). Hence, the impairment of the formation of $ONOO^-$ by the scavenging of NO• by DPTVP becomes very important.

We observed the effects of $ONOO^-$ in the protein carbonylation assay. We observed that the NO• donor, SNP, induced oxidation of the proteins of mice`s liver, kidney and brain, increasing the product of this oxidation, the carbonyls content. These effects are probably due to the formation of $ONOO^-$ from the reaction between the released NO• and $O_2\bullet^-$, which is constantly produced by mitochondrial respiration (Murphy, 2009). $ONOO^-$ can react with susceptible amino acids such as lysine, cysteine and arginine and oxidize them. (Singh *et al.*, 2007). We observed that DPTVP was able to protect against the protein oxidation induced by SNP, which could indicate a scavenger activity against $ONOO^-$. The high antioxidant activity of DPTVP against $ONOO^-$ might be explained by the observations with other tellurides in the literature. Briviba et al. (1998) demonstrated that several tellurides have the ability to scavenge peroxynitrite, and to regenerate using RSH (Briviba *et al.*, 1998). The mechanism is very similar to the observed with H_2O_2, by an oxygen transference and formation of telluroxide.

Nevertheless, SPN can also release the ferricyanide anion $[(CN)_5–Fe]^{-3}$ that can react with H_2O_2 via Fenton reaction, generating OH• (Rauhala *et al.*, 1998). Despite

DPTVP also has the ability to scavenge this radical, it is believed that the ferricyanide anion is released after the NO and under light exposure, (Loiacono e Beart, 1992).

Another finding which cannot be unnoticed is the difference between the antioxidant concentration with and without tissue. We have previously reported that very low concentrations such as 2 µM can decrease lipid peroxidation in rat brain, kidney and liver homogenates (De Avila *et al.*, 2006; Avila *et al.*, 2008). Here, we observed that to decrease protein oxidation a concentration of approximately 3 µM is already efficient. Nevertheless, we observed that to reach the scavenger ROS an RNS activity, DPTVP was needed at higher concentrations, such as 50µM. This suggests that probably the components present in the tissues homogenates can react with the compound and generate a metabolite which has better activity than the DPTVP by itself. The tissue influence on the antioxidant activity of the compounds is an important matter, once it can also cause a decrease in the scavenger potency (Puntel *et al.*, 2009).

Organotellurium compounds are advantageous alternatives for numerous synthetic operations (Petragnani, 1994; Zeni *et al.*, 2006) and promising alternatives in the pharmacological field (Nogueira, Zeni *et al.*, 2004; Savegnago *et al.*, 2006; Avila *et al.*, 2008; Borges *et al.*, 2008), mainly due their antioxidant and scavenger activities, as demonstrated here for the DPTVP. Our results indicate that the antioxidant activity provided by DPTVP previously reported by our group (De Avila *et al.*, 2006; Avila *et al.*, 2008) is due to its scavenger activity against different ROS and RNS, probably by the formation of telluroxide. Furthermore, the potency of this scavenger activity is probably dependent on the presence of tissue components in the reaction medium. However, more studies are necessary to clarify the mechanism by which DPTVP and its metabolites can scavenge the ROS and RNS.

Figure Legends

Figure 1: Chemical structure of DPTVP.

Figure 2: Scavenger activity of DPTVP against H_2O_2 in the CL method. Results are represented as percentage of control. The mean control value is 902392 ± 110098 cpm. Data are expressed as mean ± S.E.M (n=4).* indicates statistical difference from control group by one-way ANOVA, following by Duncan post-hoc test (p<0.05).

Figure 3: Effects of DPTVP on deoxyribose degradation. The (O) indicates basal conditions, (▼) Fe^{2+} 5 μM, (◊) H_2O_2 500μM and (■)Fe^{2+} 5 μM+ H_2O_2 500μM, the Fenton reaction (FR). Results are indicated as percentage of FR. 100% of FR value is 1.102 ± 0.009 μmol MDA/g of deoxyribose. Data are expressed as mean ± S.E.M (n=5)* indicates statistical difference from basal conditions without DPTVP # indicates statistical difference from H_2O_2 500μM without DPTVP and § indicates statistical difference from FR without DPTVP by one-way ANOVA, following by Duncan post-hoc test (p<0.05) for five individual experiments.

Figure 4: Effects of DPTVP on the DPPH• scavenging assay. The values are expressed in percentage of inhibition in relation to control without DPTVP. The mean control value is 0.959± 0.02 of absorbance (518nm). Data are expressed as mean ± S.E.M (n=4). * indicates statistical difference with p<0.05 and # refers to significance with p<0.001 by Duncan test.

Figure 5: Effects of DPTVP on the NO• scavenging assay. The values are expressed in percentage of inhibition. The mean control value is 22.3± 0.05 μM of nitrate. Data are expressed as mean ± S.E.M (n=4). * indicates statistical difference with p<0.05 by Duncan test.

Figure 6: Effects of the vinylic telluride on basal (□) and SNP- induced (■) protein carbonylation *in vitro* in liver (A), kidney (B), brain (C) of mice. Results are expressed as percent of control. 100% of control corresponds to 24.67 ± 2.4 nmols carbonyls/mg of tissue of tissue (A) to 15.9 ± 2.3 nmols carbonyls/mg of tissue of tissue (B) and 13.9 ±1.5 nmols carbonyls/mg of tissue of tissue (C). Data are expressed as mean ± S.E.M (n=4). *indicates statistical difference from the basal without DPTVP, p<0.05; ** indicates

statistical difference from SNP-induced carbonylation without DPTVP ($p<0.05$) by Duncan post-hoc test.

Figure 1

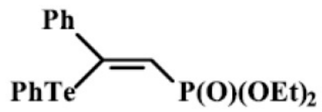


Figure 2

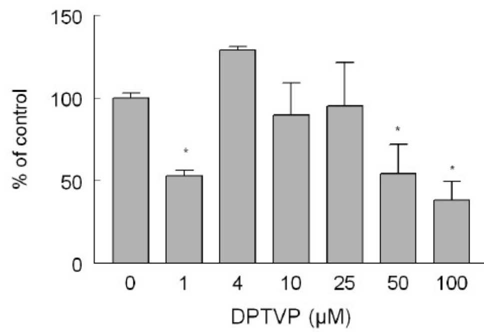


Figure 3

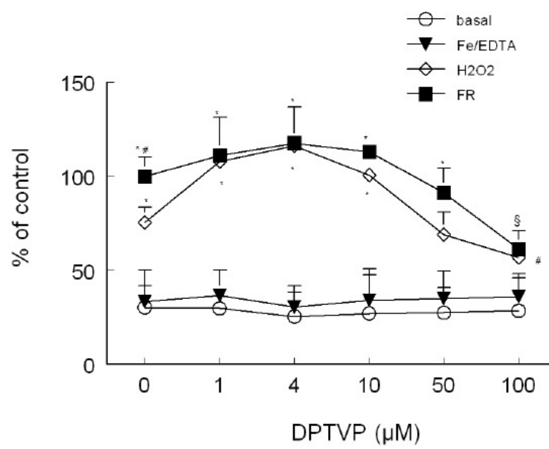


Figure 4

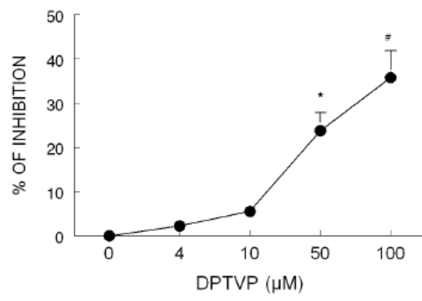


Figure 5

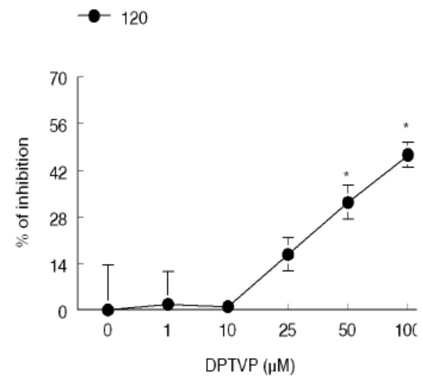
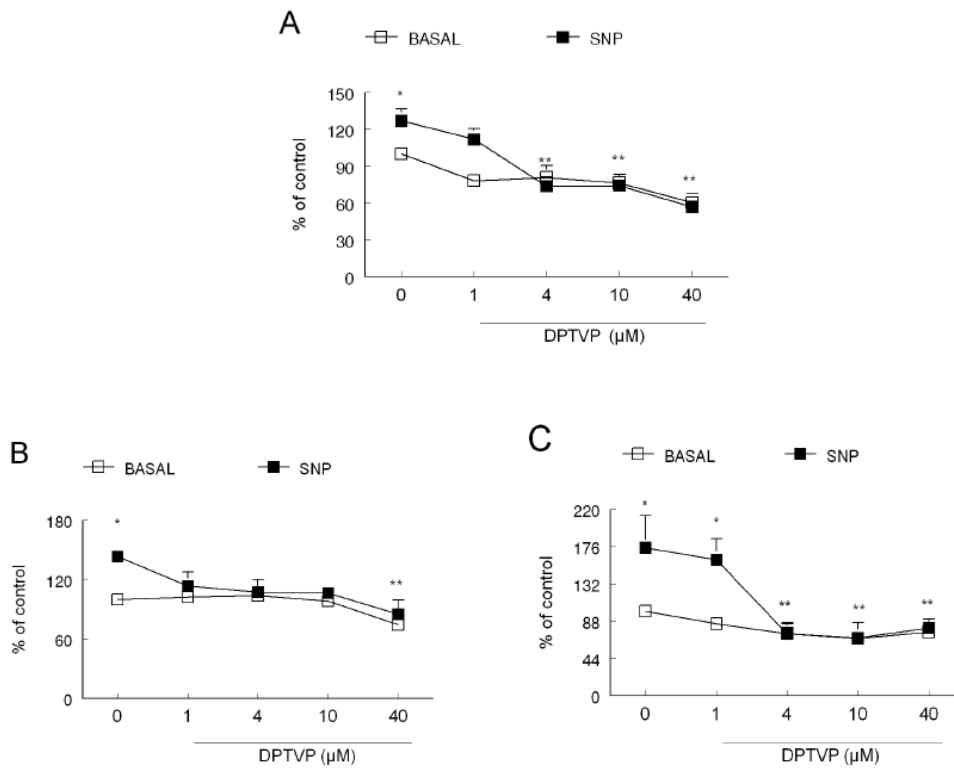


Figure 6



References

Andersson, C. M., Brattsand, R., Hallberg, A., Engman, L., Persson, J., Moldeus, P., and Cotgreave, I. (1994). Diaryl tellurides as inhibitors of lipid peroxidation in biological and chemical systems. *Free Radic Res* **20**, 401-410.

Avila, D. S., Gubert, P., Dalla Corte, C. L., Alves, D., Nogueira, C. W., Rocha, J. B., and Soares, F. A. (2007). A biochemical and toxicological study with diethyl 2-phenyl-2-tellurophenyl vinylphosphonate in a sub-chronic intraperitoneal treatment in mice. *Life Sci* **80**, 1865-1872.

Avila, D. S., Gubert, P., Palma, A., Colle, D., Alves, D., Nogueira, C. W., Rocha, J. B., and Soares, F. A. (2008). An organotellurium compound with antioxidant activity against excitotoxic agents without neurotoxic effects in brain of rats. *Brain Res Bull* **76**, 114-123.

Avila, D. S., Palma, A. S., Colle, D., Scolari, R., Manarin, F., Silveira A.F., Nogueira C.W., Rocha, J. B. T., and Soares, F. A. A. (2009). Hepatoprotector activity of a vinylic telluride against acute exposure to acetaminophen. *unpublished manuscript*.

Borges, V. C., Savegnago, L., Pinton, S., Jesse, C. R., Alves, D., and Nogueira, C. W. (2008). Vinylic telluride derivatives as promising pharmacological compounds with low toxicity. *J Appl Toxicol* **28**, 839-848.

Braga, A. L., Alberto, E. E., Soares, L. C., Rocha, J. B., Sudati, J. H., and Roos, D. H. (2009). Synthesis of telluroamino acid derivatives with remarkable GPx like activity. *Org Biomol Chem* **7**, 43-45.

Braga, A. L., Alves, E. F., Silveira, C. C., and Andrade, L. H. (2000). Stereoselective addition of sodium organyl chalcogenolates to alkynylphosphonates: synthesis of diethyl 2-(organyl)-2-(organochalcogenyl)vinylphosphonates. *Tetrahedron Letter* **41**, 161-163.

Briviba, K., Tamler, R., Klotz, L. O., Engman, L., Cotgreave, I. A., and Sies, H. (1998). Protection by organotellurium compounds against peroxynitrite-mediated oxidation and nitration reactions. *Biochem Pharmacol* **55**, 817-823.

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

de Avila, D. S., Beque, M. C., Folmer, V., Braga, A. L., Zeni, G., Nogueira, C. W., Soares, F. A., and Rocha, J. B. (2006). Diethyl 2-phenyl-2 tellurophenyl vinylphosphonate: an organotellurium compound with low toxicity. *Toxicology* **224**, 100-107.

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

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

Engman, L., Stern, D., and Pelcman, M. (1994). Thiol peroxidase of diorganoyl tellurides. *Journal of Organic Chemistry* **59**, 1973-1979.

Giokas, D. L., Vlessidis, A. G., and Evmiridis, N. P. (2007). On-line selective detection of antioxidants free-radical scavenging activity based on Co(II)/EDTA-induced luminol chemiluminescence by flow injection analysis. *Anal Chim Acta* **589**, 59-65.

Goodrum, J. F. (1998). Role of organotellurium species in tellurium neuropathy. *Neurochem Res* **23**, 1313-1319.
Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R. (1982). Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal Biochem* **126**, 131-138.
Gutteridge, J. M. (1981). Thiobarbituric acid-reactivity following iron-dependent free-radical damage to amino acids and carbohydrates. *FEBS Lett* **128**, 343-346.
Gutteridge, J. M. (1987). Ferrous-salt-promoted damage to deoxyribose and benzoate. The increased effectiveness of hydroxyl-radical scavengers in the presence of EDTA. *Biochem J* **243**, 709-714.
Halliwell, B., and Gutteridge, J. M. (1981). Formation of thiobarbituric-acid-reactive substance from deoxyribose in the presence of iron salts: the role of superoxide and hydroxyl radicals. *FEBS Lett* **128**, 347-352.
Halliwell, B., and Gutteridge, J. M. (1984). Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem J* **219**, 1-14.
Halliwell, B., and Gutterridge, J. M. C. (2003). *Free radicals in biology and medicine*. Oxford University Press.
Laden, B. P., and Porter, T. D. (2001). Inhibition of human squalene monooxygenase by tellurium compounds: evidence of interaction with vicinal sulfhydryls. *J Lipid Res* **42**, 235-240.
Levine, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, I., Lenz, A. G., Ahn, B. W., Shaltiel, S., and Stadtman, E. R. (1990). Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol* **186**, 464-478.
Loiacono, R. E., and Beart, P. M. (1992). Hippocampal lesions induced by microinjection of the nitric oxide donor nitroprusside. *Eur J Pharmacol* **216**, 331-333.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265-275.
Mandelker, L. (2008). Introduction to oxidative stress and mitochondrial dysfunction. *Vet Clin North Am Small Anim Pract* **38**, 1-30, v.
Marcocci, L., Maguire, J. J., Droy-Lefaix, M. T., and Packer, L. (1994). The nitric oxide-scavenging properties of Ginkgo biloba extract EGb 761. *Biochem Biophys Res Commun* **201**, 748-755.
Murphy, M. P. (2009). How mitochondria produce reactive oxygen species. *Biochem J* **417**, 1-13.
Nogueira, C. W., Zeni, G., and Rocha, J. B. (2004). Organoselenium and organotellurium compounds: toxicology and pharmacology. *Chem Rev* **104**, 6255-6285.
Pereira, R. P., Fachinetto, R., de Souza Prestes, A., Puntel, R. L., Santos da Silva, G. N., Heinzmann, B. M., Boschetti, T. K., Athayde, M. L., Burger, M. E., Morel, A. F., Morsch, V. M., and Rocha, J. B. (2008). Antioxidant Effects of Different Extracts from Melissa officinalis, Matricaria recutita and Cymbopogon citratus. *Neurochem Res*.
Petragnani, N. (1994). *Tellurium in organic synthesis*. Academic Press, New York.
Puntel, G. O., de Carvalho, N. R., Gubert, P., Palma, A. S., Dalla Corte, C. L., Avila, D. S., Pereira, M. E., Carratu, V. S., Bresolin, L., da Rocha, J. B., and Soares, F. A. (2009). Butane-2,3-dionethiosemicarbazone: an oxime with antioxidant properties. *Chem Biol Interact* **177**, 153-160.

Puntel, R. L., Nogueira, C. W., and Rocha, J. B. (2005). Krebs cycle intermediates modulate thiobarbituric acid reactive species (TBARS) production in rat brain in vitro. *Neurochem Res* **30**, 225-235.

Rauhala, P., Khaldi, A., Mohanakumar, K. P., and Chiueh, C. C. (1998). Apparent role of hydroxyl radicals in oxidative brain injury induced by sodium nitroprusside. *Free Radic Biol Med* **24**, 1065-1073.

Ren, X., Xue, Y., Zhang, K., Liu, J., Luo, G., Zheng, J., Mu, Y., and Shen, J. (2001). A novel dicyclodextrinyl ditelluride compound with antioxidant activity. *FEBS Lett* **507**, 377-380.

Rooseboom, M., Vermeulen, N. P., Durgut, F., and Commandeur, J. N. (2002). Comparative study on the bioactivation mechanisms and cytotoxicity of Te-phenyl-L-tellurocysteine, Se-phenyl-L-selenocysteine, and S-phenyl-L-cysteine. *Chem Res Toxicol* **15**, 1610-1618.

Savegnago, L., Borges, V. C., Alves, D., Jesse, C. R., Rocha, J. B., and Nogueira, C. W. (2006). Evaluation of antioxidant activity and potential toxicity of 1-buthyltelurenyl-2-methylthioheptene. *Life Sci* **79**, 1546-1552.

Schiar, V. P., Dos Santos, D. B., Paixao, M. W., Nogueira, C. W., Rocha, J. B., and Zeni, G. (2009). Human erythrocyte hemolysis induced by selenium and tellurium compounds increased by GSH or glucose: a possible involvement of reactive oxygen species. *Chem Biol Interact* **177**, 28-33.

Singh, I. N., Sullivan, P. G., and Hall, E. D. (2007). Peroxynitrite-mediated oxidative damage to brain mitochondria: Protective effects of peroxynitrite scavengers. *J Neurosci Res* **85**, 2216-2223.

Singh, N. P., McCoy, M. T., Tice, R. R., and Schneider, E. L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* **175**, 184-191.

Southorn, P. A., and Powis, G. (1988). Free radicals in medicine. I. Chemical nature and biologic reactions. *Mayo Clin Proc* **63**, 381-389.

Souza, A. C., Luchese, C., Santos Neto, J. S., and Nogueira, C. W. (2009). Antioxidant effect of a novel class of telluroacetilene compounds: Studies in vitro and in vivo. *Life Sci*.

Sudati, J. H., Fachinetto, R., Pereira, R. P., Boligon, A. A., Athayde, M. L., Soares, F. A., de Vargas Barbosa, N. B., and Rocha, J. B. (2009). In vitro Antioxidant Activity of Valeriana officinalis Against Different Neurotoxic Agents. *Neurochem Res*.

Toews, A. D., Roe, E. B., Goodrum, J. F., Bouldin, T. W., Weaver, J., Goines, N. D., and Morell, P. (1997). Tellurium causes dose-dependent coordinate down-regulation of myelin gene expression. *Brain Res Mol Brain Res* **49**, 113-119.

Wagner, C., Fachinetto, R., Dalla Corte, C. L., Brito, V. B., Severo, D., de Oliveira Costa Dias, G., Morel, A. F., Nogueira, C. W., and Rocha, J. B. (2006). Quercitrin, a glycoside form of quercetin, prevents lipid peroxidation in vitro. *Brain Res* **1107**, 192-198.

Zeni, G., Ludtke, D. S., Panatieri, R. B., and Braga, A. L. (2006). Vinylic tellurides: from preparation to their applicability in organic synthesis. *Chem Rev* **106**, 1032-1076.

V. Discussão

Compostos orgânicos de telúrio têm sido descritos como bons antioxidantes em diversos modelos animais em ensaios in vitro e ex vivo (Engman *et al.*, 1995; Briviba *et al.*, 1998; Engman *et al.*, 2000; Tiano *et al.*, 2000; Cunha *et al.*, 2005; Souza *et al.*, 2009b). Entretanto, alguns falham em prover total benefício devido aos efeitos tóxicos que podem causar (Toews *et al.*, 1997; Laden e Porter, 2001; Nogueira *et al.*, 2001; Borges *et al.*, 2005). Por outro lado, dentre as diversas classes de compostos, os teluretos vinílicos têm sido reportados como de baixa toxicidade e como compostos promissores para avaliações farmacológicas (Savegnago *et al.*, 2006; Borges *et al.*, 2008). Dentre os teluretos vinilicos, o DPTVP se destaca pela baixa toxicidade quando administrado sub-agudamente e por vias de rápida absorção em camundongos e pela potente atividade antioxidante, conforme previamente demonstrada pelo nosso grupo (De Avila *et al.*, 2006; Avila *et al.*, 2007). O presente estudo procura demonstrar o potencial farmacológico do DPTVP como hepato e neuroprotetor e sugere que tais efeitos se devem à atividade "scavenger" ou neutralizadora de espécies reativas de oxigênio e nitrogênio.

A possível atividade neuroprotetora do DPTVP foi desafiada in vitro utilizando-se dois agentes pró-oxidantes que mimetizam os efeitos causados pelo glutamato em excesso na fenda sináptica, o QA e o SNP (Artigo 1). O QA, um metabólito endógeno neuroativo resultante do metabolismo das quinureninas, possui um efeito agonista em receptores NMDA (Stone, 1991). A toxicidade por QA é mediada pela ativação do receptor NMDA levando ao aumento de Ca^{2+} intracelular, estresse oxidativo, depleção de ATP, disfunção mitocondrial e dano celular (Akaike *et al.*, 1999). Em nosso trabalho, foi observado que o QA causou um significativo aumento nos níveis de TBARS em homogeneizados de cérebro total e das estruturas cerebrais utilizadas (córtex, hipocampo e estriado). De acordo com a literatura, o QA induz peroxidação lipídica em cérebro de ratos (Rios e Santamaria, 1991) e a lipoperoxidação pode ser reduzida por antioxidantes (Rossato *et al.*, 2002). Da mesma maneira, o DPTVP também foi capaz de reduzir a lipoperoxidação causada pelo QA na concentração de aproximadamente 2µM

em todas as estruturas testadas. Apesar dos conhecidos efeitos do QA sobre a mitocôndria, não foi observado nenhuma alteração na viabilidade mitocondrial em fatias de córtex, hipocampo e estriado. Provavelmente a concentração de QA utilizada no ensaio não foi suficiente para causar danos à mitocôndria, apenas a produção de ERO.

O influxo de Ca^{2+} é uma etapa fundamental na excitotoxicidade por glutamato, uma vez que toda a cascata de produção de ERO é desencadeada pelo aumento de sua concentração intracelular (Akaike *et al.*, 1999). Um dos seus efeitos é ativar a NOS, uma enzima dependente de Ca^{2+}/calmodulina (Beckman *et al.*, 1990), produzindo NO. Este, por sua vez, pode gerar facilmente, na presença de $O_2^{\bullet -}$, o $ONOO^-$ (Beckman *et al.*, 1990). Para mimetizar esses efeitos, foi utilizado o SNP, uma molécula que libera lentamente NO para o meio. Conforme a literatura (ref), o SNP induziu aumento na lipoperoxidação em todas as estruturas cerebrais testadas, bem como no cérebro total. Além disso, a viabilidade mitocondrial de córtex, hipocampo e estriado foi significativamente reduzida pelo tratamento com SNP, indicando um dano acentuado aos neurônios dessas regiões. Novamente, o DPTVP protegeu contra a peroxidação lipídica, dessa vez induzida pelo SNP. A concentração média antioxidante entre as estruturas testadas foi 3µM. A viabilidade mitocondrial também foi restaurada pelo telureto vinílico, entretanto, de uma maneira mais eficiente no córtex (~4µM) do que no hipocampo (~40 µM) e no estriado (efeito bifásico: 4µM e depois a partir de 40µM). É importante ressaltar que o DPTVP não causou disfunção mitocondrial nas concentrações testadas, excluindo um possível efeito neurotóxico deste composto nas concentrações usadas

O SNC é um órgão-alvo para a toxicidade dos compostos orgânicos de telúrio. Por interagirem com grupamentos –SH essenciais à algumas enzimas, levam à redução na atividade destas e graves alterações ao SNC. Alguns compostos inibem a esqualeno monooxigenase, uma enzima tiólica essencial à síntese de mielina (Toews *et al.*, 1991; Toews *et al.*, 1997; Laden e Porter, 2001). O ditelureto de difenila inibe a Na^+/K^+ ATPase, causando alterações na permeabilidade das membranas neuronais (Borges *et al.*, 2005). Estudos prévios realizados por Nogueira et al. (2001, 2002) demonstraram que o ditelureto de difenila diminui a união especifica the [^{3}H]glutamato em membranas e

diminui a captação de $[^3H]$glutamato em sinaptossomas de roedores, sendo este efeito atribuído à oxidação de grupos –SH dos receptores NMDA e dos transportadores EAAT. Entretanto, diferentemente do ditelureto, o DPTVP não causou alterações significativas ao sistema glutamatérgico. A captação de $[^3H]$glutamato foi reduzida em fatias de córtex e hipocampo em concentrações muito maiores do que as concentrações antioxidantes (<5µM) e não foi alterada no estriado. A concentração a aprtir da qual o DPTVP reduziu a captação de $[^3H]$glutamato foi 100µM, cerca de 10 vezes maior do que a encontrada para o ditelureto nas mesmas condições experimentais (Nogueira *et al.*, 2002). A liberação de $[^3H]$glutamato em sinaptossomas também só foi aumentada em 100µM. Além disso, o binding de $[^3H]$glutamato em membranas foi reduzido em 50% a partir da concentração de 50µM de DPTVP, ainda muito acima da concentração capaz de reduzir em 50% a lipoperoxidação lipídica induzida por SNP ou QA. A necessidade de altas concentrações de DPTVP para alterar a captação, liberação e ligação de $[^3H]$ glutamato provavelmente se deve à fraca interação do composto com grupamentos -SH. Em estudos prévios, nosso grupo observou que era necessária uma alta concentração do composto (120µM) para oxidar o DTT, um composto ditiólico simples sem qualquer impedimento alostérico como ocorre em enzimas (De Avila *et al.*, 2006). Além disso, uma redução da enzima sulfidrílica δ-ALA D em cérebro de camundongos in vitro só foi obtida a partir de 120µM, porém, não foi inibida após tratamento crônico com o telureto vinílico (De Avila *et al.*, 2006).

Com a ausência de alterações severas ao sistema glutamatérgico e a promissora neuroproteção in vitro, o DPTVP foi testado in vivo. Foi utilizado um modelo de exposição ambiental ao Mn, o qual causou alterações comportamentais e nos parâmetros bioquímicos avaliados (Manuscrito 1).

A exposição ocupacional e ambiental ao Mn pode levar a uma condição denominada *locura manganica* ou manganismo, uma desordem caracterizada por déficit motor (Couper, 1837b). O controle motor é principalmente coordenado por áreas ricas em neurônios dopaminérgicos, como o estriado (Sloot e Gramsbergen, 1994). O estriado é uma área cerebral com alta expressão de transportador de metais divalentes tipo 1 (DMT-1), responsável pela captação de Mn através da barreira sangue-cérebro (Au *et*

al., 2008). De fato, em nosso estudo foram observadas alterações motoras nos ratos expostos ao Mn e, ex vivo, estresse oxidativo e redução na captação de [^{3}H]glutamato apenas no estriado, dados consistentes com o acúmulo de Mn encontrado nessa estrutura.

Intracelularmente, o Mn se acumula na mitocôndria (~100nM), causando colapso no potencial da membrana e problemas na produção de energia (Gavin *et al.*, 1992). Além disso, o Mn inibe diretamente os complexos da cadeia transportadora de elétrons (Malecki, 2001), reduzindo a produção de ATP e aumentado a produção de ERO. Esses efeitos foram observados pela viabilidade mitocondrial reduzida e pela lipoperoxidação aumentada no estriado dos ratos expostos ao Mn. Já o tratamento por duas semanas pela via i.p. com DPTVP reverteu o dano mitocondrial causado provavelmente pelo estresse oxidativo que também havia aumentado a lipoperoxidação. Esses achados sugerem que o telureto vinílico também apresenta ação antioxidante in vivo, da mesma maneira como observado in vitro.

A reduzida captação de [^{3}H]glutamato no estriado dos animais após 4 meses de exposição ao Mn foi um achado bastante interessante. Anteriormente, tal efeito só havia sido descrito in vitro. (Erikson e Aschner, 2002) demonstraram que a expressão do RNAm do transportador de glutamato (GLAST) mostrou-se reduzida pelo Mn em culturas de astrócitos. Além disso (Mutkus *et al.*, 2005), demonstraram em células ovarianas de hamster que o Mn inibia a captação de [D-2,3- ^{3}H]D-aspartato através dos transportadores GLAST (EAAT-1) e GLT-1(EAAT-2), sugerindo que o mesmo ocorreria no SNC. Como esses transportadores possuem resíduos de cisteína essenciais à sua funcionalidade (Trotti *et al.*, 1998), a oxidação destes em função do tratamento com Mn levaria à redução da remoção do glutamato da fenda sináptica, causando excitotoxicidade aos neurônios glutamatérgicos no estriado. Acredita-se que esses efeitos sejam causados devido à geração de ERO nos astrócitos pelo Mn. Como os astrócitos são células gliais responsáveis por regular a concentração de neurotransmissores e de outras substâncias químicas na fenda sináptica, eles acumulam todo o Mn que entra no SNC (Pentschew *et al.*, 1963). Ao se acumular na mitocôndria dos astrócitos, o Mn desencadeia a produção de ERO, as quais oxidam os

grupamentos –SH dos transportadores de glutamato. Essa hipótese se ajusta pelo fato de o tratamento com DPTVP, cujo potencial antioxidante já havia sido observado nos ensaios de TBARS e MTT, ter recuperado a captação de [^{3}H]glutamato que havia sido prejudicada pelo Mn.

Entretanto, um outro achado bastante interessante pode fornecer uma segunda hipótese em relação mecanismo neuroprotetor do DPTVP: os níveis de Mn no estriado dos animais tratados com Mn e DPTVP semelhantes aos dos animais do grupo controle. Não existem dados na literatura sobre o transporte de Te^{2+} através da barreira sangue – cérebro para o SNC e como este poderia afetar o transporte de Mn^{2+}. Porém, alguns estudos com telurito (Te^{3+}) mostraram que este pode ser captado via sistema de transporte de monocarboxilato (Borghese *et al.*, 2008), o mesmo que capta Mn (Aschner *et al.*, 2007). Alternativamente, o Te^{3+} também pode ser transportado aravés de um processo pH dependente , possivelmente via DMT-1, uma vez que esse transportador é próton-dependente (Borsetti *et al.*, 2003). Esses dados são inconclusivos em relação ao que ocorre com o Te^{2+}, entretanto devem ser considerados em futuros estudos sobre o transporte desse metal no SNC.

A fim de pesquisar outra possível atividade farmacológica do DPTVP, a atividade hepatoprotetora foi avaliada (Manuscrito 2). O modelo de hepatotoxicidade utilizado foi o APAP. O APAP é um analgésico/antipirético amplamente utilizado, entretando seu uso abusivo vem sendo relacionado a casos de falência hepática, transplantes de fígado e em tentativas de suicídio (Watson *et al.*, 2003; Larson *et al.*, 2005; Nourjah *et al.*, 2006).

A depleção de –SH não protéico (~90% GSH) após a admnistração de APAP, foi observada quando utilizadas uma dose média (200mg/Kg i.p.) ou elevada (300mg/Kg) do agente hepatotóxico. Tal efeito é bem descrito na literatura, uma vez que o metabólito NAPQI, produzido pela superdosagem de APAP, reage rapidamente com a GSH intracelular (Nelson, 1990). A redução na concentração de GSH acaba causando um desbalanço entre a produção normal de ROS e o sistema antioxidante intracelular, uma vez que a GSH é essencial para a atividade da GPX (Larson *et al.*, 2005). Como em um efeito em cascata, as ROS geram a produção de mais ERO, iniciando os danos oxidativos às células (Larson, 2007), como a lipoperoxidação elevada no fígado dos

animais tratados com ambas as doses de APAP. Também foi observado aumento na atividade sérica da ALT, uma enzima presente no citosol de hepatócitos (Sallie *et al.*, 1991). A liberação dessa enzima na corrente sanguínea indica dano à estrutura dos hepatócitos, o que foi evidenciado pela análise histopatológica.

A administração de doses diferentes de APAP surtiu efeitos semelhantes, porém em diferentes escalas de intensidade. Dessa maneira, o DPTVP foi bastante eficaz como hepatoprotetor quando a dose de APAP utilizada foi 200mg/Kg. As três doses testadas (30, 50 e 100µmol/Kg) reverteram a depleção de SH, a lipoperoxidação e o vazamento de ALT do fígado para a circulação, bem como auxiliou na regeneração do tecido hepático, como observado nas análises histológicas. Já contra a dose de APAP de 300mg/Kg, o DPTVP não foi tão eficaz. O dano hepático foi tão severo que mesmo a dose de DPTVP que restaurou os níveis de SH não protéico (50µmol/Kg) não recuperou o aumento de lipoperoxidação e a atividade aumentada da ALT. Entretanto, houve melhora na morfologia dos hepatócitos, uma vez que nas análises histológicas das secções de fígado de animais tratados com DPTVP havia apenas infiltração leucocitária e não mais polimorfismo nuclear e necrose na região periportal.

A administração de APAP (200 e 300mg/Kg) também causou inibição da enzima sulfidrílica δ-ALA-D hepática, o que poderia indicar oxidação dos resíduos cisteinil do sítio ativo da enzima pelas ERO. Entretanto, o DTT, um agente redutor ditiólico, não reverteu a inibição causada pela administração de APAP, indicando que tal efeito não se deve à oxidação de SH na enzima. Esses achados estão de acordo com Rocha et al. (2005), os quais observaram o mesmo padrão de inibição pelo APAP em ratos, indicando que provavelmente a inibição da enzima pode ocorrer por um outro mecanismo ou que a oxidação dos resíduos sulfidrílicos seja tão acentuada que a reversão pelo DTT não ocorre.

No protocolo experimental utilizado, não houve alteração na atividade das enzimas antioxidantes SOD e CAT pela administração de APAP. Entretanto, alguns autores reportaram efeitos nessas enzimas apenas alguns dias após a administração de APAP (Olaleye e Rocha, 2008; Sabir e Rocha, 2008). Além disso, Cimegris et al., (2009) demonstraram em coelhos que os níveis de expressão de RNAm de SOD e CAT não

foram alterados pelo APAP administrado em uma dose que causou danos oxidativos e histológicos. Eles sugerem que a atividade dessas enzimas não possui um papel crítico na hepatotoxicidade por APAP.

As atividades hepato e neuroprotetora do DPTVP descritas no presente trabalho se devem provavelmente às propriedades antioxidantes do composto, uma vez que diversos parâmetros bioquímicos de estresse oxidativo alterados foram reparados pelo DPTVP. Entretanto, diversas são as ERO e ERN que podem ser geradas durante o estresse oxidativo. Dessa maneira, se fazia necessário verificar quais espécies poderiam ser neutralizadas pelo telureto vinílico (manuscrito 3).

O H_2O_2 é relativamente uma espécie mais estável dentre as ERO, uma vez que não possui um elétron desemparelhado, entretanto, é a maior fonte de espécies reativas intracelulares (Mandelker, 2008). É gerado por vários processos, como pelas atividades da xantina e urato oxidase e como produto final da dismutação do $O_2\bullet^-$ pela SOD, processo este mais ativo durante o estresse oxidativo (Murphy, 2009). Dessa maneira, o controle dos níveis de H_2O_2 é bastante importante durante processo patogênico, a fim de reduzir os danos intra e extracelulares. Utilizando uma técnica de quimioluminescência, foi observado que o DPTVP possui atividade “scavenger” de H_2O_2, apresentando uma curva bifásica. O efeito foi observado na concentração de 1µM, sendo observado novamente após 50 µM.

O H_2O_2 em excesso pode degradar certas hemeproteínas como a mioglobina e hemoglobina, liberando Fe^{2+} (Halliwell e Gutterridge, 2003). Este metal pode reagir com o H_2O_2 via reação de Fenton, gerando OH•. O resultado dessa reação pode ser observado utilizando-se o ensaio de degradação da desoxirribose, uma vez que os radicais OH• podem degradar este açúcar, os quais reagem com o ácido tiobarbitúrico. Foi observado que o DPTVP foi capaz de interagir com a reação de Fenton, reduzindo a formação de TBARS. Entretanto, era necessário confirmar se esse efeito se devia pela neutralização de OH•.

Assim, foi também realizado o ensaio do DPPH•. O radical DPPH• é um composto de cor púrpura o qual possui apenas um elétron desemparelhado, similarmente ao OH• (Choi *et al.*, 2002). Novamente o DPTVP mostrou-se capaz de

neutralizar esse tipo de espécie em uma concentração menor que a encontrada no ensaio de degradação da desoxirribose. Esse fato deve-se provavelmente pela ausência, no meio reacional, de agentes que continuam gerando espécies reativas, como é o caso do ensaio da degradação da desoxirribose, na qual tanto o Fe^{2+} quanto o H_2O_2 estão presentes em altas concentrações.

Tal atividade pode ser atribuída a estrutura química do DPTVP. Este possui um grupamento fosfonato, o qual é um grupo retirador de elétrons, causando uma diminuição na densidade eletrônica do átomo de Te, o qual se torna levemente eletrofílico (Te^+). Considerando que as ERO possuem um elétron desemparelhado, o Te^+ receberia este elétron, tornando-se um teluróxido (Te IV). Este mecanismo é similar ao proposto por vários autores para compostos de telúrio simétricos, como os diaril teluretos (Andersson *et al.*, 1994; Engman *et al.*, 1994; Briviba *et al.*, 1998; Engman *et al.*, 2000) como para assimétricos, como os teluretos derivados de aminoácidos (Braga *et al.*, 2009). Através deste mecanismo, explica-se a neutralização de H_2O_2 e de oxigênio singlete, incluindo os presentes nas ERN.

De fato, o DPTVP também se mostrou um bom agente oxidante contra ERN. A mais abundante ERN intracelular é o NO, o qual é uma importante molécula sinalizadora, tornando-se ameaçadora quando produzida em concentrações excessivas. Tal periculosidade se deve mais à produção de $ONOO^-$, um metabólito gerado pela reação de NO com o $O_2\bullet^-$ (Halliwell e Gutterridge, 2003). Dessa maneira, a atividade "scavenger" de NO do DPTVP determinada no presente trabalho é bastante importante.

Além disso, o composto também mostrou ser capaz de neutralizar o $ONOO^-$, conforme observado no ensaio de carbonilação de proteínas. O SNP, um doador de NO•, induziu a oxidação de proteínas em fígado, rim e cérebro de camundongos, gerando proteínas carboniladas, provavelmente pela geraçao de $ONOO^-$ no meio reacional. O conteúdo de proteínas carboniladas foi significativamente reduzido pelo DPTVP a partir da concentração média de 2 µM nos três tecidos. Esta atividade antioxidante contra o ONOO- pode ser explicada através de relatos prévios na literatura de que compostos orgânicos de Te possuem atividade neutralizadora contra essa ERN. Briviba et al. (1998) demonstraram que vários teluretos são capazes de neutralizar o

ONOO- e se regenerar utilizando grupamentos tiólicos presentes no tecido (conforme Figura 2). O mecanismo é muito similar ao descrito para as ERO, através de uma transferência de oxigênio e a formação de teluróxido.

Além disso, o SNP também pode liberar o ânion ferricianeto $[(CN)_5–Fe]^{-3}$, o qual pode reagir com o H_2O_2 através da reação de Fenton, gerando OH• (Rauhala *et al.*, 1998). Apesar de o DPTVP também possuir a habilidade de neutralizar esse radical, acredita-se que o ânion ferricianeto é liberado após o NO• e sob exposição à luz (Loiacono e Beart, 1992), o que reinforça a hipótese de que a carbonilaçao de proteínas ocorreu principalemente em virtude da formação de $ONOO^-$.

Uma observação bastante interessante é em relação às concentrações antioxidantes encontradas nos ensaios na ausência e na presença de tecido. Quando há tecido no meio, a concentração de DPTVP necessária para reduzir a lipoperoxidação ou a carbonilação induzida por algum agente pró-oxidante é menor do que a necessária para a atividade "scavenger" sem tecido. Esse achado sugere que provavelmente a presença de componentes teciduais como enzimas podem reagir com o composto, gerando um metabólito com atividade antioxidante melhor do que a do próprio DPTVP.

Dessa maneira, o presente estudo evidencia os possíveis efeitos benéficos que podem ser obtidos pela administração de DPTVP em doses baixas, como neuro e hepatoprotetor. Além disso, o trabalho reinforça a importância de agentes antioxidantes no tratamento de doenças nas quais o estresse oxidativo está envolvido. Estudos mais aprofundados sobre o DPTVP ainda devem ser realizados, a fim de elucidar o mecanismo de ação antioxidante e se as atividades farmacológicas do composto se devem a ele próprio ou a um metabólito.

VI. Conclusões

1) O DPTVP apresentou uma potente atividade antioxidante frente aos agentes pró-oxidantes SNP e QA homogeneizados de cérebro total, córtex, hipocampo e estriado, além de ter protegido contra a disfunção

mitocondrial induzida por SNP nessas estruturas, indicando possível atividade neuroprotetora *in vitro*;

2) O DPTVP não alterou a captação, liberação e união específica de $[^3H]$ glutamato nas concentrações antioxidantes, demonstrando baixa toxicidade ao SNC;

3) A administração crônica de $MnCl_2$ (137mg/Kg) na água de beber de ratos causou redução na atividade exploratória e motora dos animais, e o co-tratamento com DPTVP por duas semanas (0,150µmol/Kg, i.p) reverteu parcialmente esses efeitos, melhorando a performance dos ratos nos testes;

4) O co-tratamento com DPTVP reverteu os danos causados no estriado dos ratos expostos cronicamente ao Mn, reduzindo os níveis de TBARS, aumentando a viabilidade mitocondrial, normalizando captação de $[^3H]$ glutamato e os níveis de Mn nessa estrutura;

5) O DPTVP foi capaz de reverter os danos hepáticos causados pelo APAP (200mg/Kg) revertendo a depleção de tióis não protéicos, normalizando os níveis de TBARS, a atividade sérica da ALT e as alterações histomorfológicas causadas pelo agente hepatotóxico; entretanto, apenas os danos observados histologicamente foram recuperados contra dose de 300mg/Kg de APAP;

6) O DPTVP mostrou atuar como agente neutralizador de ERO e ERN, evidenciando um mecanismo para a atividade antioxidante do composto; além disso, sugere-se que a ação “scavenger” ocorra via formação de teluróxido e que provavelmente um metabólito do composto seja mais ativo como antioxidante do que ele por si só.

VII. Perspectivas

1. Avaliar outras possíveis atividades farmacológicas atribuíveis ao DPTVP. Atualmente vêm sendo avaliada a atividade antidepressiva do composto;
2. Averiguar os metabólitos do DPTVP formados após a sua administração em roedores;
3. Estudar os mecanismos envolvidos no transporte de Te no SNC e sua influência no transporte de Mn;

VIII.Referências Bibliográficas

AIKENS, J. e DIX, T. A. Perhydroxyl radical (HOO.) initiated lipid peroxidation. The role of fatty acid hydroperoxides. **J Biol Chem**, v.266, n.23, Aug 15, p.15091-8. 1991.

AKAIKE, A., KATSUKI, H., KUME, T. e MAEDA, T. Reactive oxygen species in NMDA receptor-mediated glutamate neurotoxicity. **Parkinsonism Relat Disord**, v.5, n.4, Dec, p.203-7. 1999.

ANDERSSON, B. S., RUNDGREN, M., NELSON, S. D. e HARDER, S. N-acetyl-p-benzoquinone imine-induced changes in the energy metabolism in hepatocytes. **Chem Biol Interact**, v.75, n.2, p.201-11. 1990.

ANDERSSON, C. M., BRATTSAND, R., HALLBERG, A., ENGMAN, L., PERSSON, J., MOLDEUS, P. e COTGREAVE, I. Diaryl tellurides as inhibitors of lipid peroxidation in biological and chemical systems. **Free Radic Res**, v.20, n.6, Jun, p.401-10. 1994.

ANDERSSON, C. M., HALLBERG, A., BRATTSAND, R., COTGREAVE, I., ENGMAN, L. e PERSSON, J. Glutathione peroxidase-like activity of diaryl tellurides. **Bioorganic Medicine ans Chemistry Letters**, v.3, p.2553-2558. 1993.

ARCHIBALD, F. S. e TYREE, C. Manganese poisoning and the attack of trivalent manganese upon catecholamines. **Arch Biochem Biophys**, v.256, n.2, Aug 1, p.638-50. 1987.

ASCHNER, M. e DORMAN, D. C. Manganese: pharmacokinetics and molecular mechanisms of brain uptake. **Toxicol Rev**, v.25, n.3, p.147-54. 2006.

ASCHNER, M., GANNON, M. e KIMELBERG, H. K. Manganese uptake and efflux in cultured rat astrocytes. **J Neurochem**, v.58, n.2, Feb, p.730-5. 1992.

ASCHNER, M., GUILARTE, T. R., SCHNEIDER, J. S. e ZHENG, W. Manganese: recent advances in understanding its transport and neurotoxicity. **Toxicol Appl Pharmacol**, v.221, n.2, Jun 1, p.131-47. 2007.

AU, C., BENEDETTO, A. e ASCHNER, M. Manganese transport in eukaryotes: the role of DMT1. **Neurotoxicology**, v.29, n.4, Jul, p.569-76. 2008.

AVILA, D. S., GUBERT, P., DALLA CORTE, C. L., ALVES, D., NOGUEIRA, C. W., ROCHA, J. B. e SOARES, F. A. A biochemical and toxicological study with diethyl 2-phenyl-2-tellurophenyl vinylphosphonate in a sub-chronic intraperitoneal treatment in mice. **Life Sci**, v.80, n.20, Apr 24, p.1865-72. 2007.

BAEZ, S., LINDERSON, Y. e SEGURA-AGUILAR, J. Superoxide dismutase and catalase enhance autoxidation during one-electron reduction of aminochrome by NADPH-cytochrome P-450 reductase. **Biochem Mol Med**, v.54, n.1, Feb, p.12-8. 1995.

BARBOSA, N. B., ROCHA, J. B., ZENI, G., EMANUELLI, T., BEQUE, M. C. e BRAGA, A. L. Effect of organic forms of selenium on delta-aminolevulinate dehydratase from liver, kidney, and brain of adult rats. **Toxicol Appl Pharmacol**, v.149, n.2, Apr, p.243-53. 1998.

BATES, J. N., BAKER, M. T., GUERRA, R., JR. e HARRISON, D. G. Nitric oxide generation from nitroprusside by vascular tissue. Evidence that reduction of the nitroprusside anion and cyanide loss are required. **Biochem Pharmacol**, v.42 Suppl, Dec 11, p.S157-65. 1991.

BECHARA, E. J. H., M.H.G., M., MONTEIRO, H. P., HERMES-LIMA, M., PEREIRA, B., DEMASI, M., COSTA, C. A., ADBALLA, D. S. P., ONUKI, J., WENDEL, C. M. A. e MASCIO, P. D. A free radical hypothesis of lead poisoning and inborn porphyrias associated with 5-aminolevulinic acid overload. **Quimica Nova**, v.16, p.385-392. 1993.

BECKMAN, J. S., BECKMAN, T. W., CHEN, J., MARSHALL, P. A. e FREEMAN, B. A. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. **Proc Natl Acad Sci U S A**, v.87, n.4, Feb, p.1620-4. 1990.

BJORKMAN, R. Central antinociceptive effects of non-steroidal anti-inflammatory drugs and paracetamol. Experimental studies in the rat. **Acta Anaesthesiol Scand Suppl**, v.103, p.1-44. 1995.

BLAIS, F. X., ONISCHUK, R. T. e DEMEIO, R. H. Hemolysis by tellurite. I. The tellurite test for hemolysis. **J Am Osteopath Assoc**, v.72, n.2, Oct, p.207-10. 1972.

BORGES, V. C., ROCHA, J. B. e NOGUEIRA, C. W. Effect of diphenyl diselenide, diphenyl ditelluride and ebselen on cerebral Na(+), K(+)-ATPase activity in rats. **Toxicology**, v.215, n.3, Nov 15, p.191-7. 2005.

BORGES, V. C., SAVEGNAGO, L., PINTON, S., JESSE, C. R., ALVES, D. e NOGUEIRA, C. W. Vinylic telluride derivatives as promising pharmacological compounds with low toxicity. **J Appl Toxicol**, v.28, n.7, Oct, p.839-48. 2008.

BORGHESE, R., MARCHETTI, D. e ZANNONI, D. The highly toxic oxyanion tellurite (TeO (3) (2-)) enters the phototrophic bacterium Rhodobacter capsulatus via an as yet uncharacterized monocarboxylate transport system. **Arch Microbiol**, v.189, n.2, Feb, p.93-100. 2008.

BORSETTI, F., TONINELLO, A. e ZANNONI, D. Tellurite uptake by cells of the facultative phototroph Rhodobacter capsulatus is a Delta pH-dependent process. **FEBS Lett**, v.554, n.3, Nov 20, p.315-8. 2003.

BOVERIS, A. e CHANCE, B. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. **Biochem J**, v.134, n.3, Jul, p.707-16. 1973.

BRAGA, A. L., ALBERTO, E. E., SOARES, L. C., ROCHA, J. B., SUDATI, J. H. e ROOS, D. H. Synthesis of telluroamino acid derivatives with remarkable GPx like activity. **Org Biomol Chem**, v.7, n.1, Jan 7, p.43-5. 2009.

BRAMBILLA, D., MANCUSO, C., SCUDERI, M. R., BOSCO, P., CANTARELLA, G., LEMPEREUR, L., DI BENEDETTO, G., PEZZINO, S. e BERNARDINI, R. The role of antioxidant supplement in immune system, neoplastic, and neurodegenerative disorders: a point of view for an assessment of the risk/benefit profile. **Nutr J**, v.7, p.29. 2008.

BRAUNGART, E., GERLACH, M., RIEDERER, P., BAUMEISTER, R. e HOENER, M. C. Caenorhabditis elegans MPP+ model of Parkinson's disease for high-throughput drug screenings. **Neurodegener Dis**, v.1, n.4-5, p.175-83. 2004.

BRIVIBA, K., TAMLER, R., KLOTZ, L. O., ENGMAN, L., COTGREAVE, I. A. e SIES, H. Protection by organotellurium compounds against peroxynitrite-mediated oxidation and nitration reactions. **Biochem Pharmacol**, v.55, n.6, Mar 15, p.817-23. 1998.

BROUILLET, E. P., SHINOBU, L., MCGARVEY, U., HOCHBERG, F. e BEAL, M. F. Manganese injection into the rat striatum produces excitotoxic lesions by impairing energy metabolism. **Exp Neurol**, v.120, n.1, Mar, p.89-94. 1993.

BUCK, M. R., KARUSTIS, D. G., DAY, N. A., HONN, K. V. e SLOANE, B. F. Degradation of extracellular-matrix proteins by human cathepsin B from normal and tumour tissues. **Biochem J**, v.282 (Pt 1), Feb 15, p.273-8. 1992.

CALNE, D. B., CHU, N. S., HUANG, C. C., LU, C. S. e OLANOW, W. Manganism and idiopathic parkinsonism: similarities and differences. **Neurology**, v.44, n.9, Sep, p.1583-6. 1994.

CANO, G., SUAREZ-ROCA, H. e BONILLA, E. Alterations of excitatory amino acid receptors in the brain of manganese-treated mice. **Mol Chem Neuropathol**, v.30, n.1-2, Jan-Feb, p.41-52. 1997.

CHALFIE, M., TU, Y., EUSKIRCHEN, G., WARD, W. W. e PRASHER, D. C. Green fluorescent protein as a marker for gene expression. **Science**, v.263, n.5148, Feb 11, p.802-5. 1994.

CHEN, W., KOENIGS, L. L., THOMPSON, S. J., PETER, R. M., RETTIE, A. E., TRAGER, W. F. e NELSON, S. D. Oxidation of acetaminophen to its toxic quinone imine and nontoxic catechol metabolites by baculovirus-expressed and purified human cytochromes P450 2E1 and 2A6. **Chem Res Toxicol**, v.11, n.4, Apr, p.295-301. 1998.

CHOI, C. W., KIM, S. C., HWANG S.S., CHOI, B. K., AHN, H. J., LEE, M. Y., PARK, S. H. e KIM, S. K. Antioxidant activity and free radical scavenging capacity between Korean medicinal plants and flavonoids by assay-guided comparison. **Plant Sciences**, v.153, p.1161-1168. 2002.

CHRISTIANSON, D. W. Structural chemistry and biology of manganese metalloenzymes. **Prog Biophys Mol Biol**, v.67, n.2-3, p.217-52. 1997.

CIGREMIS, Y., TUREL, H., ADIGUZEL, K., AKGOZ, M., KART, A., KARAMAN, M. e OZEN, H. The effects of acute acetaminophen toxicity on hepatic mRNA expression of SOD, CAT, GSH-Px, and levels of peroxynitrite, nitric oxide, reduced glutathione, and malondialdehyde in rabbit. **Mol Cell Biochem**, v.323, n.1-2, Mar, p.31-8. 2009.

COLE, R. D., ANDERSON, G. L. e WILLIAMS, P. L. The nematode Caenorhabditis elegans as a model of organophosphate-induced mammalian neurotoxicity. **Toxicol Appl Pharmacol**, v.194, n.3, Feb 1, p.248-56. 2004.

COMASSETO, J. V., LING, L. W., STEFANI, H. A. e PETRAGNANI, N. Vinylic selenides and tellurides: preparation, reactivity ad synthetic applications. **Synthesis**, v.4, p.373. 1997.

COMMITTEE ON INFECTIOUS DISEASES, C. Aspirin and Reye syndrome. Committee on Infectious Diseases. **Pediatrics**, v.69, n.6, Jun, p.810-2. 1982.

COUPER, J. On the effects of black oxide of manganese when inhaled into the lungs. **British Annals of Medicine and Pharmacology**, v.1, p.41-42. 1837a.

CUNHA, R. L., URANO, M. E., CHAGAS, J. R., ALMEIDA, P. C., BINCOLETTO, C., TERSARIOL, I. L. e COMASSETO, J. V. Tellurium-based cysteine protease inhibitors: evaluation of novel organotellurium(IV) compounds as inhibitors of human cathepsin B. **Bioorg Med Chem Lett**, v.15, n.3, Feb 1, p.755-60. 2005.

DANBOLT, N. C. Glutamate uptake. **Prog Neurobiol**, v.65, n.1, Sep, p.1-105. 2001.

DAVIDSON, D. G. e EASTHAM, W. N. Acute liver necrosis following overdose of paracetamol. **Br Med J**, v.2, n.5512, Aug 27, p.497-9. 1966.

DE AVILA, D. S., BEQUE, M. C., FOLMER, V., BRAGA, A. L., ZENI, G., NOGUEIRA, C. W., SOARES, F. A. e ROCHA, J. B. Diethyl 2-phenyl-2 tellurophenyl vinylphosphonate: an organotellurium compound with low toxicity. **Toxicology**, v.224, n.1-2, Jul 5, p.100-7. 2006.

DEUTICKE, B., LUTKEMEIER, P. e POSER, B. Tellurite-induced damage of the erythrocyte membrane. Manifestations and mechanisms. **Biochim Biophys Acta**, v.1109, n.1, Aug 10, p.97-107. 1992.

DOBSON, A. W., ERIKSON, K. M. e ASCHNER, M. Manganese neurotoxicity. **Ann N Y Acad Sci**, v.1012, Mar, p.115-28. 2004.

DODD, S., DEAN, O., COPOLOV, D. L., MALHI, G. S. e BERK, M. N-acetylcysteine for antioxidant therapy: pharmacology and clinical utility. **Expert Opin Biol Ther**, v.8, n.12, Dec, p.1955-62. 2008.

DORMAN, D. C., STRUVE, M. F., VITARELLA, D., BYERLY, F. L., GOETZ, J. e MILLER, R. Neurotoxicity of manganese chloride in neonatal and adult CD rats following subchronic (21-day) high-dose oral exposure. **J Appl Toxicol**, v.20, n.3, May-Jun, p.179-87. 2000.

DROGE, W. Free radicals in the physiological control of cell function. **Physiol Rev**, v.82, n.1, Jan, p.47-95. 2002.

EMANUELLI, T., PAGEL, F. W., ALVES, L. B., REGNER, A. e SOUZA, D. O. Inhibition of adenylate cyclase activity by 5-aminolevulinic acid in rat and human brain. **Neurochem Int**, v.38, n.3, Mar, p.213-8. 2001.

ENGMAN, L., AL-MAHARIK, N., MCNAUGHTON, M., BIRMINGHAM, A. e POWIS, G. Thioredoxin reductase and cancer cell growth inhibition by organotellurium compounds that could be selectively incorporated into tumor cells. **Bioorg Med Chem**, v.11, n.23, Nov 17, p.5091-100. 2003.

ENGMAN, L., KANDRA, T., GALLEGOS, A., WILLIAMS, R. e POWIS, G. Water-soluble organotellurium compounds inhibit thioredoxin reductase and the growth of human cancer cells. **Anticancer Drug Des**, v.15, n.5, Oct, p.323-30. 2000.

ENGMAN, L., PERSSON, J., VESSMAN, K., EKSTROM, M., BERGLUND, M. e ANDERSSON, C. M. Organotellurium compounds as efficient retarders of lipid peroxidation in methanol. **Free Radic Biol Med**, v.19, n.4, Oct, p.441-52. 1995.

ENGMAN, L., STERN, D. e PELCMAN, M. Thiol peroxidase of diorganoyl tellurides. **Journal of Organic Chemistry**, v.59, p.1973-1979. 1994.

ERIKSON, K. e ASCHNER, M. Manganese causes differential regulation of glutamate transporter (GLAST) taurine transporter and metallothionein in cultured rat astrocytes. **Neurotoxicology**, v.23, n.4-5, Oct, p.595-602. 2002.

ERIKSON, K. M. e ASCHNER, M. Manganese neurotoxicity and glutamate-GABA interaction. **Neurochem Int**, v.43, n.4-5, Sep-Oct, p.475-80. 2003.

FAIRHILL, L. T. **Tellurium**. In: (Ed.). Industrial Toxicology. New York: Hafner Publishing Co, 1969. Tellurium

FINLEY, J. W., JOHNSON, P. E. e JOHNSON, L. K. Sex affects manganese absorption and retention by humans from a diet adequate in manganese. **Am J Clin Nutr**, v.60, n.6, Dec, p.949-55. 1994.

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

FONTANA, R. J. Acute liver failure due to drugs. **Semin Liver Dis**, v.28, n.2, May, p.175-87. 2008.

FORREST, J. A., CLEMENTS, J. A. e PRESCOTT, L. F. Clinical pharmacokinetics of paracetamol. **Clin Pharmacokinet**, v.7, n.2, Mar-Apr, p.93-107. 1982.

GAVIN, C. E., GUNTER, K. K. e GUNTER, T. E. Mn2+ sequestration by mitochondria and inhibition of oxidative phosphorylation. **Toxicol Appl Pharmacol**, v.115, n.1, Jul, p.1-5. 1992.

GAVIN, C. E., GUNTER, K. K. e GUNTER, T. E. Manganese and calcium transport in mitochondria: implications for manganese toxicity. **Neurotoxicology**, v.20, n.2-3, Apr-Jun, p.445-53. 1999.

GERBER, G. B., LEONARD, A. e HANTSON, P. Carcinogenicity, mutagenicity and teratogenicity of manganese compounds. **Crit Rev Oncol Hematol**, v.42, n.1, Apr, p.25-34. 2002.

GIBSON, J. D., PUMFORD, N. R., SAMOKYSZYN, V. M. e HINSON, J. A. Mechanism of acetaminophen-induced hepatotoxicity: covalent binding versus oxidative stress. **Chem Res Toxicol**, v.9, n.3, Apr-May, p.580-5. 1996.

GOODRUM, J. F. Role of organotellurium species in tellurium neuropathy. **Neurochem Res**, v.23, n.10, Oct, p.1313-9. 1998.

GRAF, E., MAHONEY, J. R., BRYANT, R. G. e EATON, J. W. Iron-catalyzed hydroxyl radical formation. Stringent requirement for free iron coordination site. **J Biol Chem**, v.259, n.6, Mar 25, p.3620-4. 1984.

GREEN, D. R. e REED, J. C. Mitochondria and apoptosis. **Science**, v.281, n.5381, Aug 28, p.1309-12. 1998.

GREEN, L. C., WAGNER, D. A., GLOGOWSKI, J., SKIPPER, P. L., WISHNOK, J. S. e TANNENBAUM, S. R. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. **Anal Biochem**, v.126, n.1, Oct, p.131-8. 1982.

GREENE, J. G. e GREENAMYRE, J. T. Bioenergetics and glutamate excitotoxicity. **Prog Neurobiol**, v.48, n.6, Apr, p.613-34. 1996.

GREGER, J. L. Dietary standards for manganese: overlap between nutritional and toxicological studies. **J Nutr**, v.128, n.2 Suppl, Feb, p.368S-371S. 1998.

GROGAN, T. M., FENOGLIO-PRIESER, C., ZEHEB, R., BELLAMY, W., FRUTIGER, Y., VELA, E., STEMMERMAN, G., MACDONALD, J., RICHTER, L., GALLEGOS, A. e POWIS, G. Thioredoxin, a putative oncogene product, is overexpressed in gastric carcinoma and associated with increased proliferation and increased cell survival. **Hum Pathol**, v.31, n.4, Apr, p.475-81. 2000.

HABER, F. e WEISS, J. On the catalysis of hydroperoxide. **Naturwissenschaften**, v.20, p.948-950. 1932.

HALLIWELL, B. Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. **Drugs Aging**, v.18, n.9, p.685-716. 2001.

HALLIWELL, B. e ARUOMA, O. I. DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems. **FEBS Lett**, v.281, n.1-2, Apr 9, p.9-19. 1991.

HALLIWELL, B. e CHIRICO, S. Lipid peroxidation: its mechanism, measurement, and significance. **Am J Clin Nutr**, v.57, n.5 Suppl, May, p.715S-724S; discussion 724S-725S. 1993.

HALLIWELL, B. e GUTTERIDGE, J. M. Formation of thiobarbituric-acid-reactive substance from deoxyribose in the presence of iron salts: the role of superoxide and hydroxyl radicals. **FEBS Lett**, v.128, n.2, Jun 15, p.347-52. 1981.

HALLIWELL, B. e GUTTERIDGE, J. M. Oxygen toxicity, oxygen radicals, transition metals and disease. **Biochem J**, v.219, n.1, Apr 1, p.1-14. 1984.

HALLIWELL, B. e GUTTERRIDGE, J. M. C. **Free radicals in biology and medicine**: Oxford University Press. 2003

HALLIWELL, B., ZHAO, K. e WHITEMAN, M. Nitric oxide and peroxynitrite. The ugly, the uglier and the not so good: a personal view of recent controversies. **Free Radic Res**, v.31, n.6, Dec, p.651-69. 1999.

HARMAN, A. W., KYLE, M. E., SERRONI, A. e FARBER, J. L. The killing of cultured hepatocytes by N-acetyl-p-benzoquinone imine (NAPQI) as a model of the cytotoxicity of acetaminophen. **Biochem Pharmacol**, v.41, n.8, Apr 15, p.1111-7. 1991.

HAZELL, A. S. e NORENBERG, M. D. Manganese decreases glutamate uptake in cultured astrocytes. **Neurochem Res**, v.22, n.12, Dec, p.1443-7. 1997.

HELMCKE, K. J., AVILA, D. S. e ASCHNER, M. Utility of Caenorhabditis elegans in high throughput neurotoxicological research. **Neurotoxicol Teratol**, Dec 7. 2008.

HOPE, I. A. **Background oc Caenorhabiditis elegans**. In: (Ed.). C.elegans: a Practical Approach. New York: Oxford University Press, 1999. Background oc Caenorhabiditis elegans, p.1-15

HSU, C. C., LIN, C. C., LIAO, T. S. e YIN, M. C. Protective effect of s-allyl cysteine and s-propyl cysteine on acetaminophen-induced hepatotoxicity in mice. **Food Chem Toxicol**, v.44, n.3, Mar, p.393-7. 2006.

JACOB, C., ARTEEL, G. E., KANDA, T., ENGMAN, L. e SIES, H. Water-soluble organotellurium compounds: catalytic protection against peroxynitrite and release of zinc from metallothionein. **Chem Res Toxicol**, v.13, n.1, Jan, p.3-9. 2000.

JAESCHKE, H. e BAJT, M. L. Intracellular signaling mechanisms of acetaminophen-induced liver cell death. **Toxicol Sci**, v.89, n.1, Jan, p.31-41. 2006.

JAESCHKE, H., KNIGHT, T. R. e BAJT, M. L. The role of oxidant stress and reactive nitrogen species in acetaminophen hepatotoxicity. **Toxicol Lett**, v.144, n.3, Oct 15, p.279-88. 2003.

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

KALETTA, T. e HENGARTNER, M. O. Finding function in novel targets: C. elegans as a model organism. **Nat Rev Drug Discov**, v.5, n.5, May, p.387-98. 2006.

KANDA, T., ENGMAN, L., COTGREAVE, I. A. e POWIS, G. Novel Water-Soluble Diorganyl Tellurides with Thiol Peroxidase and Antioxidant Activity. **J Org Chem**, v.64, n.22, Oct 29, p.8161-8169. 1999.

KANSKI, J., DRAKE, J., AKSENOVA, M., ENGMAN, L. e BUTTERFIELD, D. A. Antioxidant activity of the organotellurium compound 3-[4-(N,N-dimethylamino)benzenetellurenyl]propanesulfonic acid against oxidative stress in synaptosomal membrane systems and neuronal cultures. **Brain Res**, v.911, n.1, Aug 17, p.12-21. 2001.

KASS, G. E. Mitochondrial involvement in drug-induced hepatic injury. **Chem Biol Interact**, v.163, n.1-2, Oct 27, p.145-59. 2006.

KEEN, C. L., ENSUNSA, J. L., WATSON, M. H., BALY, D. L., DONOVAN, S. M., MONACO, M. H. e CLEGG, M. S. Nutritional aspects of manganese from experimental studies. **Neurotoxicology**, v.20, n.2-3, Apr-Jun, p.213-23. 1999.

KLAMAN, D. **Organotellurium compounds**. In: (Ed.). Methods of Organic Chemistry. New York: George Thieme Verlag Stuttgart, 1990. Organotellurium compounds

KLUGER, M. J. Fever: role of pyrogens and cryogens. **Physiol Rev**, v.71, n.1, Jan, p.93-127. 1991.

KON, K., KIM, J. S., JAESCHKE, H. e LEMASTERS, J. J. Mitochondrial permeability transition in acetaminophen-induced necrosis and apoptosis of cultured mouse hepatocytes. **Hepatology**, v.40, n.5, Nov, p.1170-9. 2004.

KOZENITZKY, L., DAVID, M., SREDAI, B., ALBECK, M. e SHOHAT, B. Immunomodulatory effects of AS101 on interleukin-2 production and T-lymphocyte function of lymphocytes treated with psoralens and ultraviolet A. **Photodermatol Photoimmunol Photomed**, v.9, n.1, Feb, p.24-8. 1992.

KRIEGLSTEIN, J. Excitotoxicity and neuroprotection. **European Journal of Pharmaceutical Sciences**, v.5, p.181-187. 1997.

KVAMME, E. Synthesis of glutamate and its regulation. **Prog Brain Res**, v.116, p.73-85. 1998.

LADEN, B. P. e PORTER, T. D. Inhibition of human squalene monooxygenase by tellurium compounds: evidence of interaction with vicinal sulfhydryls. **J Lipid Res**, v.42, n.2, Feb, p.235-40. 2001.

LARSON, A. M. Acetaminophen hepatotoxicity. **Clin Liver Dis**, v.11, n.3, Aug, p.525-48, vi. 2007.

LARSON, A. M., POLSON, J., FONTANA, R. J., DAVERN, T. J., LALANI, E., HYNAN, L. S., REISCH, J. S., SCHIODT, F. V., OSTAPOWICZ, G., SHAKIL, A. O. e LEE, W. M. Acetaminophen-induced acute liver failure: results of a United States multicenter, prospective study. **Hepatology**, v.42, n.6, Dec, p.1364-72. 2005.

LINDEN, C. H. e RUMACK, B. H. Acetaminophen overdose. **Emerg Med Clin North Am**, v.2, n.1, Feb, p.103-19. 1984.

LOIACONO, R. E. e BEART, P. M. Hippocampal lesions induced by microinjection of the nitric oxide donor nitroprusside. **Eur J Pharmacol**, v.216, n.2, Jun 5, p.331-3. 1992.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. e RANDALL, R. J. Protein measurement with the Folin phenol reagent. **J Biol Chem**, v.193, n.1, Nov, p.265-75. 1951.

MABAN, L. K. e ESCOTT-STUMP, S. **Alimentos, Nutrição e Dietoterapia**. Rio de Janeiro: Roca. 1998

MACIEL, E. N., BOLZAN, R. C., BRAGA, A. L. e ROCHA, J. B. Diphenyl diselenide and diphenyl ditelluride differentially affect delta-aminolevulinate dehydratase from liver, kidney, and brain of mice. **J Biochem Mol Toxicol**, v.14, n.6, p.310-9. 2000.

MAKIN, A. e WILLIAMS, R. The current management of paracetamol overdosage. **Br J Clin Pract**, v.48, n.3, May-Jun, p.144-8. 1994.

MALECKI, E. A. Manganese toxicity is associated with mitochondrial dysfunction and DNA fragmentation in rat primary striatal neurons. **Brain Res Bull**, v.55, n.2, May 15, p.225-8. 2001.

MANDELKER, L. Introduction to oxidative stress and mitochondrial dysfunction. **Vet Clin North Am Small Anim Pract**, v.38, n.1, Jan, p.1-30, v. 2008.

MANYIKE, P. T., KHARASCH, E. D., KALHORN, T. F. e SLATTERY, J. T. Contribution of CYP2E1 and CYP3A to acetaminophen reactive metabolite formation. **Clin Pharmacol Ther**, v.67, n.3, Mar, p.275-82. 2000.

MARCOCCI, L., MAGUIRE, J. J., DROY-LEFAIX, M. T. e PACKER, L. The nitric oxide-scavenging properties of Ginkgo biloba extract EGb 761. **Biochem Biophys Res Commun**, v.201, n.2, Jun 15, p.748-55. 1994.

MCJUNKIN, B., BARWICK, K. W., LITTLE, W. C. e WINFIELD, J. B. Fatal massive hepatic necrosis following acetaminophen overdose. **JAMA**, v.236, n.16, Oct 18, p.1874-5. 1976.

MELDRUM, B. S., AKBAR, M. T. e CHAPMAN, A. G. Glutamate receptors and transporters in genetic and acquired models of epilepsy. **Epilepsy Res**, v.36, n.2-3, Sep, p.189-204. 1999.

MEOTTI, F. C., BORGES, V. C., ZENI, G., ROCHA, J. B. e NOGUEIRA, C. W. Potential renal and hepatic toxicity of diphenyl diselenide, diphenyl ditelluride and Ebselen for rats and mice. **Toxicol Lett**, v.143, n.1, Jun 5, p.9-16. 2003.

MILATOVIC, D., YIN, Z., GUPTA, R. C., SIDORYK, M., ALBRECHT, J., ASCHNER, J. L. e ASCHNER, M. Manganese induces oxidative impairment in cultured rat astrocytes. **Toxicol Sci**, v.98, n.1, Jul, p.198-205. 2007.

MINAMI, T. e MOTOYOSHIYA, J. Vinylphosphonate in organic synthesis. **Synthesis**, v.3, p.333-349. 1992.

MISRA, H. P. e FRIDOVICH, I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. **J Biol Chem**, v.247, n.10, May 25, p.3170-5. 1972.

MITCHELL, J. R., JOLLOW, D. J., POTTER, W. Z., DAVIS, D. C., GILLETTE, J. R. e BRODIE, B. B. Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. **J Pharmacol Exp Ther**, v.187, n.1, Oct, p.185-94. 1973.

MURPHY, M. P. How mitochondria produce reactive oxygen species. **Biochem J**, v.417, n.1, Jan 1, p.1-13. 2009.

MUTKUS, L., ASCHNER, J. L., FITSANAKIS, V. e ASCHNER, M. The in vitro uptake of glutamate in GLAST and GLT-1 transfected mutant CHO-K1 cells is inhibited by manganese. **Biol Trace Elem Res**, v.107, n.3, Dec, p.221-30. 2005.

NELSON, S. D. Molecular mechanisms of the hepatotoxicity caused by acetaminophen. **Semin Liver Dis**, v.10, n.4, Nov, p.267-78. 1990.

NEWLAND, M. C. Animal models of manganese's neurotoxicity. **Neurotoxicology**, v.20, n.2-3, Apr-Jun, p.415-32. 1999.

NOGUEIRA, C. W., BORGES, V. C., ZENI, G. e ROCHA, J. B. Organochalcogens effects on delta-aminolevulinate dehydratase activity from human erythrocytic cells in vitro. **Toxicology**, v.191, n.2-3, Sep 30, p.169-78. 2003.

NOGUEIRA, C. W., MEOTTI, F. C., CURTE, E., PILISSAO, C., ZENI, G. e ROCHA, J. B. Investigations into the potential neurotoxicity induced by diselenides in mice and rats. **Toxicology**, v.183, n.1-3, Feb 1, p.29-37. 2003.

NOGUEIRA, C. W., ROTTA, L. N., PERRY, M. L., SOUZA, D. O. e DA ROCHA, J. B. Diphenyl diselenide and diphenyl ditelluride affect the rat glutamatergic system in vitro and in vivo. **Brain Res**, v.906, n.1-2, Jul 6, p.157-63. 2001.

NOGUEIRA, C. W., ROTTA, L. N., ZENI, G., SOUZA, D. O. e ROCHA, J. B. Exposure to ebselen changes glutamate uptake and release by rat brain synaptosomes. **Neurochem Res**, v.27, n.4, Apr, p.283-8. 2002.

NOGUEIRA, C. W., ZENI, G. e ROCHA, J. B. Organoselenium and organotellurium compounds: toxicology and pharmacology. **Chem Rev**, v.104, n.12, Dec, p.6255-85. 2004.

NOURJAH, P., AHMAD, S. R., KARWOSKI, C. e WILLY, M. Estimates of acetaminophen (Paracetomal)-associated overdoses in the United States. **Pharmacoepidemiol Drug Saf**, v.15, n.6, Jun, p.398-405. 2006.

OLALEYE, M. T. e ROCHA, B. T. Acetaminophen-induced liver damage in mice: effects of some medicinal plants on the oxidative defense system. **Exp Toxicol Pathol**, v.59, n.5, Mar, p.319-27. 2008.

PENTSCHEW, A., EBNER, F. F. e KOVATCH, R. M. Experimental Manganese Encephalopathy in Monkeys. A Preliminary Report. **J Neuropathol Exp Neurol**, v.22, Jul, p.488-99. 1963.

PFEIFER, G. D., ROPER, J. M., DORMAN, D. e LYNAM, D. R. Health and environmental testing of manganese exhaust products from use of methylcyclopentadienyl manganese tricarbonyl in gasoline. **Sci Total Environ**, v.334-335, Dec 1, p.397-408. 2004.

PRESCOTT, L. F. Paracetamol overdosage. Pharmacological considerations and clinical management. **Drugs**, v.25, n.3, Mar, p.290-314. 1983.

PUMFORD, N. R., HINSON, J. A., POTTER, D. W., ROWLAND, K. L., BENSON, R. W. e ROBERTS, D. W. Immunochemical quantitation of 3-(cystein-S-yl)acetaminophen adducts in serum and liver proteins of acetaminophen-treated mice. **J Pharmacol Exp Ther**, v.248, n.1, Jan, p.190-6. 1989.

PUNTEL, R. L., NOGUEIRA, C. W. e ROCHA, J. B. Krebs cycle intermediates modulate thiobarbituric acid reactive species (TBARS) production in rat brain in vitro. **Neurochem Res**, v.30, n.2, Feb, p.225-35. 2005.

RAUHALA, P., KHALDI, A., MOHANAKUMAR, K. P. e CHIUEH, C. C. Apparent role of hydroxyl radicals in oxidative brain injury induced by sodium nitroprusside. **Free Radic Biol Med**, v.24, n.7-8, May, p.1065-73. 1998.

REN, X., XUE, Y., ZHANG, K., LIU, J., LUO, G., ZHENG, J., MU, Y. e SHEN, J. A novel dicyclodextrinyl ditelluride compound with antioxidant activity. **FEBS Lett**, v.507, n.3, Nov 2, p.377-80. 2001.

RIOS, C. e SANTAMARIA, A. Quinolinic acid is a potent lipid peroxidant in rat brain homogenates. **Neurochem Res**, v.16, n.10, Oct, p.1139-43. 1991.

ROBINSON, M. B. e DOWD, L. A. Heterogeneity and functional properties of subtypes of sodium-dependent glutamate transporters in the mammalian central nervous system. **Adv Pharmacol**, v.37, p.69-115. 1997.

ROCHA, J. B. T., GABRIEL, D., ZENI, G., POSSER, T., SIQUEIRA, L., NOGUEIRA, C. W. e FOLMER, V. Ebselen and diphenyl diselenide change biochemical hepatic responses to overdosage with paracetamol. **Environmental Toxicology and Pharmacology**, v.19, n.2, p.255-261. 2005.

ROH, J. Y., LEE, J. e CHOI, J. Assessment of stress-related gene expression in the heavy metal-exposed nematode Caenorhabditis elegans: a potential biomarker for metal-induced toxicity monitoring and environmental risk assessment. **Environ Toxicol Chem**, v.25, n.11, Nov, p.2946-56. 2006.

ROOSEBOOM, M., VERMEULEN, N. P., DURGUT, F. e COMMANDEUR, J. N. Comparative study on the bioactivation mechanisms and cytotoxicity of Te-phenyl-L-tellurocysteine, Se-phenyl-L-selenocysteine, and S-phenyl-L-cysteine. **Chem Res Toxicol**, v.15, n.12, Dec, p.1610-8. 2002.

ROSSATO, J. I., ZENI, G., MELLO, C. F., RUBIN, M. A. e ROCHA, J. B. Ebselen blocks the quinolinic acid-induced production of thiobarbituric acid reactive species but does not prevent the behavioral alterations produced by intra-striatal quinolinic acid administration in the rat. **Neurosci Lett**, v.318, n.3, Feb 1, p.137-40. 2002.

RUTLEDGE, E. M., ASCHNER, M. e KIMELBERG, H. K. Pharmacological characterization of swelling-induced D-[3H]aspartate release from primary astrocyte cultures. **Am J Physiol**, v.274, n.6 Pt 1, Jun, p.C1511-20. 1998.

SABIR, S. M. e ROCHA, J. B. Antioxidant and hepatoprotective activity of aqueous extract of Solanum fastigiatum (false "Jurubeba") against paracetamol-induced liver damage in mice. **J Ethnopharmacol**, v.120, n.2, Nov 20, p.226-32. 2008.

SALLIE, R., TREDGER, J. M. e WILLIAMS, R. Drugs and the liver. Part 1: Testing liver function. **Biopharm Drug Dispos**, v.12, n.4, May, p.251-9. 1991.

SAVEGNAGO, L., BORGES, V. C., ALVES, D., JESSE, C. R., ROCHA, J. B. e NOGUEIRA, C. W. Evaluation of antioxidant activity and potential toxicity of 1-buthyltelurenyl-2-methylthioheptene. **Life Sci**, v.79, n.16, Sep 13, p.1546-52. 2006.

SCANSETTI, G. Exposure to metals that have recently come into use. **Sci Total Environ**, v.120, n.1-2, Jun 9, p.85-91. 1992.

SCHROEDER, H. A., BUCKMAN, J. e BALASSA, J. J. Abnormal trace elements in man: tellurium. **J Chronic Dis**, v.20, n.3, Mar, p.147-61. 1967.

SEGURA-AGUILAR, J. e LIND, C. On the mechanism of the Mn3(+)-induced neurotoxicity of dopamine:prevention of quinone-derived oxygen toxicity by DT diaphorase and superoxide dismutase. **Chem Biol Interact**, v.72, n.3, p.309-24. 1989.

SEGURA-AGUILAR, J., METODIEWA, D. e BAEZ, S. The possible role of one-electron reduction of aminochrome in the neurodegenerative process of the dopaminergic system. **Neurotox Res**, v.3, n.2, Apr, p.157-65. 2001.

SLOOT, W. N. e GRAMSBERGEN, J. B. Axonal transport of manganese and its relevance to selective neurotoxicity in the rat basal ganglia. **Brain Res**, v.657, n.1-2, Sep 19, p.124-32. 1994.

SOUZA, A. C., LUCHESE, C., SANTOS NETO, J. S. e NOGUEIRA, C. W. Antioxidant effect of a novel class of telluroacetilene compounds: Studies in vitro and in vivo. **Life Sci**, v.84, n.11-12, Mar 13, p.351-7. 2009b.

SREDNI, B., CASPI, R. R., KLEIN, A., KALECHMAN, Y., DANZIGER, Y., BEN YA'AKOV, M., TAMARI, T., SHALIT, F. e ALBECK, M. A new immunomodulating compound (AS-101) with potential therapeutic application. **Nature**, v.330, n.6144, Nov 12-18, p.173-6. 1987.

STONE, T. W. Kynurenine and glycine enhance neuronal sensitivity to N-methyl-D-aspartate. **Life Sci**, v.48, n.8, p.765-72. 1991.

SULSTON, J. E. Neuronal cell lineages in the nematode Caenorhabditis elegans. **Cold Spring Harb Symp Quant Biol**, v.48 Pt 2, p.443-52. 1983.

TAKAGAKI, G. Properties of the uptake and release of glutamic acid by synaptosomes from rat cerebral cortex. **J Neurochem**, v.27, n.6, Dec, p.1417-25. 1976.

TAKAMORI, S. VGLUTs: 'exciting' times for glutamatergic research? **Neurosci Res**, v.55, n.4, Aug, p.343-51. 2006.

TAKEDA, A. Manganese action in brain function. **Brain Res Brain Res Rev**, v.41, n.1, Jan, p.79-87. 2003.

TAYLOR, A. Biochemistry of tellurium. **Biol Trace Elem Res**, v.55, n.3, Dec, p.231-9. 1996.

THOMSON, J. S. e PRESCOTT, L. F. Liver damage and impaired glucose tolerance after paracetamol overdosage. **Br Med J**, v.2, n.5512, Aug 27, p.506-7. 1966.

TIANO, L., FEDELI, D., SANTRONI, A. M., VILLARINI, M., ENGMAN, L. e FALCIONI, G. Effect of three diaryl tellurides, and an organoselenium compound in trout erythrocytes exposed to oxidative stress in vitro. **Mutat Res**, v.464, n.2, Jan 24, p.269-77. 2000.

TIMBRELL, J. **Principles of Biochemical Toxicology**. London: Taylor & Francis. 2000

TJOLSEN, A., LUND, A. e HOLE, K. Antinociceptive effect of paracetamol in rats is partly dependent on spinal serotonergic systems. **Eur J Pharmacol**, v.193, n.2, Feb 7, p.193-201. 1991.

TOEWS, A. D., ECKERMANN, C. E., ROBERSON, M. D., LEE, S. Y. e MORELL, P. Primary demyelination induced by exposure to tellurium alters mRNA levels for nerve growth factor receptor, SCIP, 2',3'-cyclic nucleotide 3'-phosphodiesterase, and myelin proteolipid protein in rat sciatic nerve. **Brain Res Mol Brain Res**, v.11, n.3-4, Oct, p.321-5. 1991.

TOEWS, A. D., ROE, E. B., GOODRUM, J. F., BOULDIN, T. W., WEAVER, J., GOINES, N. D. e MORELL, P. Tellurium causes dose-dependent coordinate down-regulation of myelin gene expression. **Brain Res Mol Brain Res**, v.49, n.1-2, Oct 3, p.113-9. 1997.

TROTTI, D., DANBOLT, N. C. e VOLTERRA, A. Glutamate transporters are oxidant-vulnerable: a molecular link between oxidative and excitotoxic neurodegeneration? **Trends Pharmacol Sci**, v.19, n.8, Aug, p.328-34. 1998.

TROTTI, D., RIZZINI, B. L., ROSSI, D., HAUGETO, O., RACAGNI, G., DANBOLT, N. C. e VOLTERRA, A. Neuronal and glial glutamate transporters possess an SH-based redox regulatory mechanism. **Eur J Neurosci**, v.9, n.6, Jun, p.1236-43. 1997.

WANG, J. D., HUANG, C. C., HWANG, Y. H., CHIANG, J. R., LIN, J. M. e CHEN, J. S. Manganese induced parkinsonism: an outbreak due to an unrepaired ventilation control system in a ferromanganese smelter. **Br J Ind Med**, v.46, n.12, Dec, p.856-9. 1989.

WATSON, W. A., LITOVITZ, T. L., RODGERS, G. C., JR., KLEIN-SCHWARTZ, W., YOUNISS, J., ROSE, S. R., BORYS, D. e MAY, M. E. 2002 annual report of the American

Association of Poison Control Centers Toxic Exposure Surveillance System. **Am J Emerg Med**, v.21, n.5, Sep, p.353-421. 2003.

WEDLER, F. C., LEY, B. W. e GRIPPO, A. A. Manganese(II) dynamics and distribution in glial cells cultured from chick cerebral cortex. **Neurochem Res**, v.14, n.11, Nov, p.1129-35. 1989.

WHITE, S. L. e RAINBOW, P. S. A preliminary study of Cu-, Cd- and Zn-binding components in the hepatopancreas of Palaemon elegans (Crustacea: Decapoda). **Comp Biochem Physiol C**, v.83, n.1, p.111-6. 1986.

WIESLANDER, E., ENGMAN, L., SVENSJO, E., ERLANSSON, M., JOHANSSON, U., LINDEN, M., ANDERSSON, C. M. e BRATTSAND, R. Antioxidative properties of organotellurium compounds in cell systems. **Biochem Pharmacol**, v.55, n.5, Mar 1, p.573-84. 1998.

WOHLER, F. Liebigs. **Annaals of Chemistry**, v.35, p.111. 1840.

YIN, Z., ASCHNER, J. L., DOS SANTOS, A. P. e ASCHNER, M. Mitochondrial-dependent manganese neurotoxicity in rat primary astrocyte cultures. **Brain Res**, v.1203, Apr 8, p.1-11. 2008.

ZENI, G., LUDTKE, D. S., PANATIERI, R. B. e BRAGA, A. L. Vinylic tellurides: from preparation to their applicability in organic synthesis. **Chem Rev**, v.106, n.3, Mar, p.1032-76. 2006.

ZHANG, S., FU, J. e ZHOU, Z. In vitro effect of manganese chloride exposure on reactive oxygen species generation and respiratory chain complexes activities of mitochondria isolated from rat brain. **Toxicol In Vitro**, v.18, n.1, Feb, p.71-7. 2004.

ZIMMERMAN, H. J. Effects of aspirin and acetaminophen on the liver. **Arch Intern Med**, v.141, n.3 Spec No, Feb 23, p.333-42. 1981.

IX. Apêndice

IX.1: Estudo desenvolvido durante o estágio de doutorando (PDEE)

Avaliação dos possíveis efeitos protetores de compostos orgânicos de selênio e telúrio sobre a neurotoxicidade induzida por manganês em C.elegans

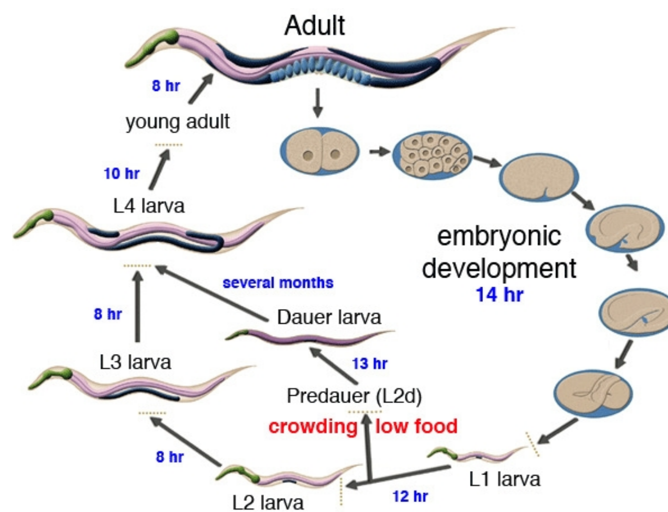
Caenorhabiditis elegans

O C.elegans é um nematódeo naturalmente presente no solo. Este invertebrado tem sido usado como um valioso modelo biológico, devido às vantagens que o seu uso provém (Helmcke *et al.*, 2008). Dentre eles, se incluem o seu tamanho reduzido, transparência, manipulação genética, rápido crescimento e desenvolvimento, alta taxa de reprodução, habilidade de sobreviverem após serem congelados e fácil aplicação de técnicas bioquímicas e de biologia molecular. Essas vantagens permitem o uso de C.elegans em sistemas in vivo com as mesmas características benéficas de ensaios in vitro (Hope, 1999).

O ciclo de vida dos C.elegans dura em torno de 21 dias, dependendo da cepa, sendo que a vida adulta inicia-se após três dias de vida (Figura 9). Os adultos põem os ovos (cerca de 200), dos quais após 12 h eclodem larvas no primeiro estágio larval (L1). Sob condições normais (alimentação irrestrita e temperatura em torno dos 20℃), as larvas vão se desenvolvendo em diferentes estágios larvais (L2, L3 e L4), tornando-se adultos capazes de oviposição (Hope, 1999). Além disso, sob restrição alimentar, os nematódeos passam para um estágio intermediário chamado Dauer, no qual podem viver num estado metabólico restrito por até três meses.

Foi descoberto que há conservação de processos fisiológicos e vias de sinalização entre C.elegans e mamíferos. Foram encontrados 60-80% de homologia genética entre humanos e os vermes (Kaletta e Hengartner, 2006). Além disso, diversos estudos têm sido conduzidos a fim de investigar a toxicidade de compostos como pesticidas (Cole *et al.*, 2004), inibidores mitocondriais (Braungart *et al.*, 2004) e metais (Roh *et al.*, 2006). Estes estudos mostraram que os valores de LD50 em vermes se correlacionam com a LD50 em roedores. Dessa maneira, o C.elegans pode ser usado

em toxicologia como um modelo de previsão de toxicidade em mamíferos. Além disso, o sistema nervoso destes nematódeos é bem caracterizado, e também se mostrou similar ao dos mamíferos (Sulston, 1983). O C.elegans possui 302 neurônios de 118 subtipos, 6393 sinapses químicas, 890 junções elétricas e 1410 junções neuromusculares (White e Rainbow, 1986). Além disso, o desenvolvimento de cepas que expressam proteínas neuronais fluorescentes permite a observação direta de dano neuronal (Chalfie *et al.*, 1994). Essa vantagem adicional amplia a ênfase dada ao C.elegans como um excelente modelo no campo toxicológico.



Ciclo de vida do C.elegans.

Inicialmente, foi realizada uma curva dose efeito a fim de se obter a LD50 para os compostos utilizados e as doses seguras que poderiam ser usadas nos próximos estudos. Para a cepa selvagem (N2), a LD_{50} do disseleneto encontrada foi de aproximadamente 45 µM ($p<0,05$, figura 1A); a do ebselen foi de aproximadamente 200µM ($p<0,05$, figura 1B) e para o DPTVP > 5mM (Figura 1C). Já para a cepa RM2702, na qual os vermes possuem uma mutação deletéria no gene que codifica o transportador de dopamina, os valores de LD_{50} foram menores quando comparados aos N2: 30µM para o disselento ($p<0,05$, Figura 2A), 120µM para o ebselen ($p<0,05$, Figura 2B) e >1mM para o DPTVP ($p<0,05$, Figura 2C).

Uma vez determinadas as doses a serem usadas, os compostos foram testados contra a toxicidade induzida por Mn. Foram realizadas curvas de dose-resposta e análises de estresse oxidativo, como a estimação de níveis de ERO e a curva de longevidade ou “life span”. As doses utilizadas de Mn foram determinadas em experimentos prévios por outros pesquisadores. Nos N2, observou-se efeitos benéficos do composto contra o Mn, principalmente por parte do ebselen e do DPTVP. O ebselen protegeu contra a toxicidade de Mn, aumentando a taxa de sobrevivência na concentração de 10µM, sendo que doses maiores causaram uma maior taxa de mortalidade quando associadas à exposição ao Mn ($p<0,05$, Figura 3B). O DPTVP também protegeu contra a toxicidade induzida por Mn na concentração de 10µM ($p<0,05$, Figura 3C), enquanto que o disseleneto não causou nenhum efeito positivo (Figura 3A). Utilizando a diclorofluoresceína diacetato (DCF-DA) como um marcador de ERO, observou-se que o estresse oxidativo está realmente envolvido com a toxicidade causada pelo Mn e que os compostos, amplamente conhecidos pelo seu potencial antioxidante, foram capazes de proteger contra a produção de ERO induzida por Mn ($p<0,05$, Figura 5A). Na figura 6A, é possível perceber que o dano oxidativo causado pelo Mn acaba levando a uma diminuição no tempo de vida de C.elegans, e que o pré-tratamento com ebselen e DPTVP aumenta a duração da vida dos vermes ao nível do controle ($p<0,05$, figura 6A).

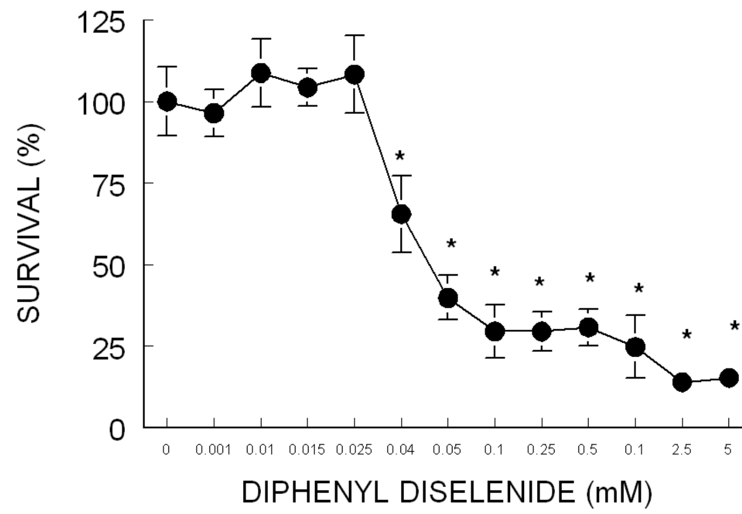
Já nos animais da cepa RM2702, doses menores de Mn foram necessárias para se causar danos similares aos observados nos selvagens. Isso se deve provavelmente à oxidação da dopamina que se encontra em excesso na fenda sináptica, uma vez que, como já mencionado, os transportadores para recaptação de dopamina não estão presentes. Como para os N2, o disseleneto não protegeu contra a mortalidade induzida por Mn. O ebselen mostrou-se eficaz na concentração de 10µM, enquanto que o DPTVP aumentou a sobrevivência na concentração de 1µM. na figura 5B pode-se observar que os níveis de ERO nos animais expostos ao Mn são maiores do que os obtidos nos N2. Entretanto, os três compostos, novamente, foram capazes de proteger contra o aumento na oxidação da DCF-DA pelas ERO. Já nos experimentos relacionados à expectativa de vida dos C.elegans, observou-se uma siginificativa redução desta nos animais expostos

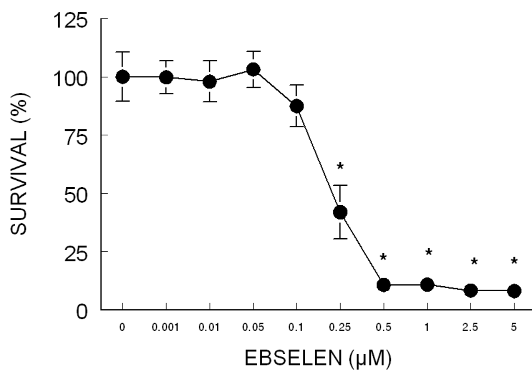
ao Mn, entretanto, os compostos reverteram esse dano, sendo que o pré-tratamento com ebselen e DPTVP gerou uma duração de vida maior do que a observada nos animais controle.

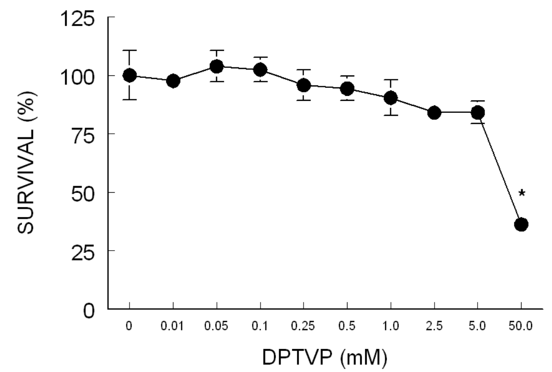
Nesse trabalho também foram utilizadas cepas alteradas geneticamente nas quais os neurônios que expresam o receptor de glutamato (glr-1::GFP) aparecem fluorescentes no microscópio de imunofluorescência. Essas observações foram realizadas a fim de se investigar os efeitos da exposição ao Mn e ao DPTVP sobre os neurônios glutamatérgicos de C.elegans. Foi observado que apenas a exposição a uma concentração muito alta de Mn (200mM) foi capaz de causar dano aos neurônios glutamatérgicos de cerca de 20% dos vermes. Como se pode observar na figura 7C, há uma degeneração axonal resultante de possível excitotoxicidade, o que não é observado nos animais controle (figura 7A) e nem mesmo nos tratados com Mn 100mM (Figura 7B). Estes resultados estão de acordo com a literatura, uma vez que está descrito que o Mn altera o sistema glutamatérgico, causando certa excitotoxicidade, sem, entretanto ser relatada morte desses neurônios (Erikson e Aschner, 2003; Milatovic *et al.*, 2007).

Outro resultado interessante foi a ausência de danos aos neurônios glutamatérgicos quando expostos ao DPTVP em altas concentrações. Pode-se observar que mesmo após 24 hs (Figura 8B) à exposição a 500µM de DPTVP os neurônios se apresentam tais quais os dos animais controle. Estes resultados corroboram com os resultados do artigo 1 desta tese, o qual reportou a baixa toxicidade do DPTVP ao sistema glutamatérgico.

Figura 1







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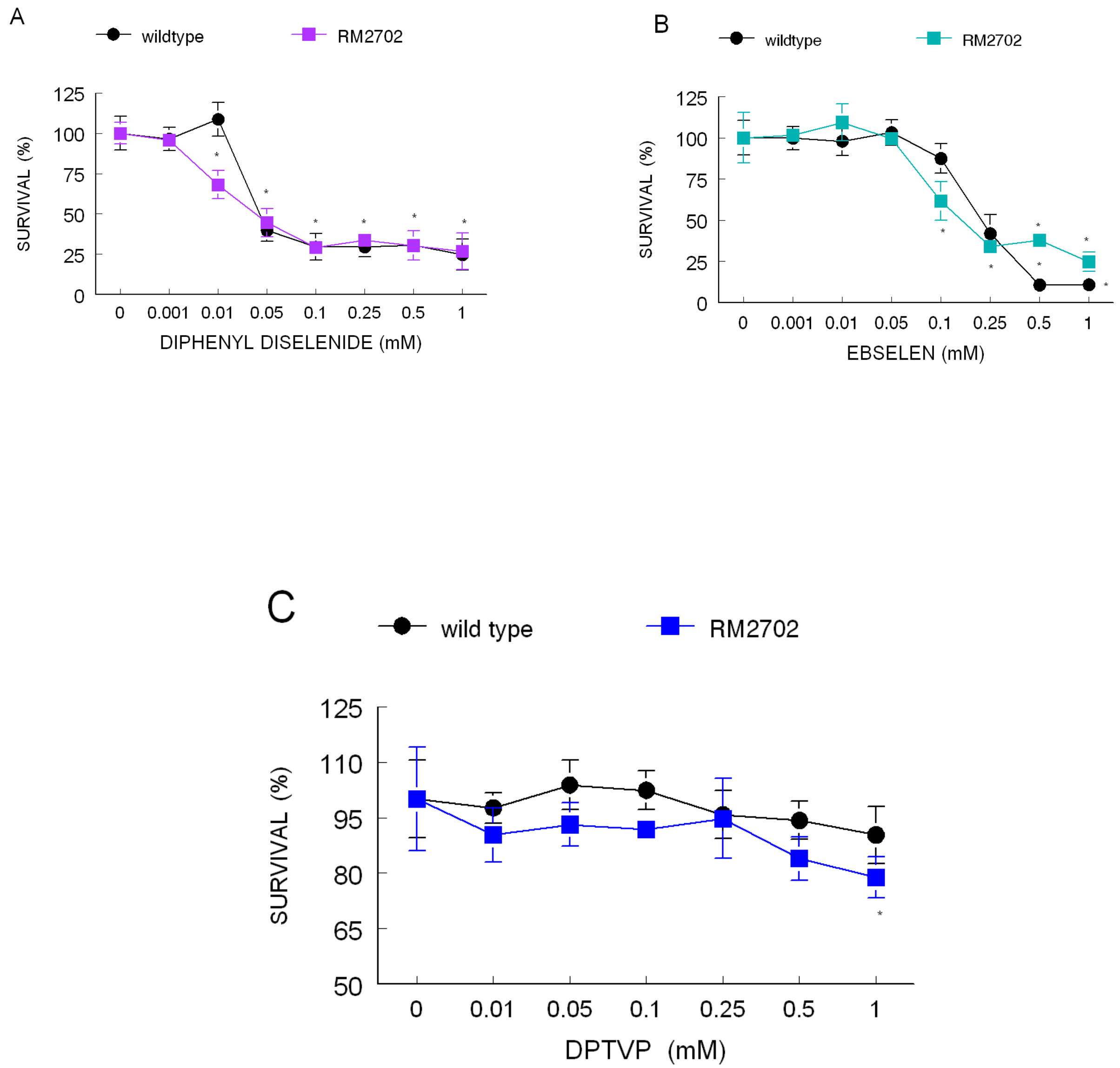
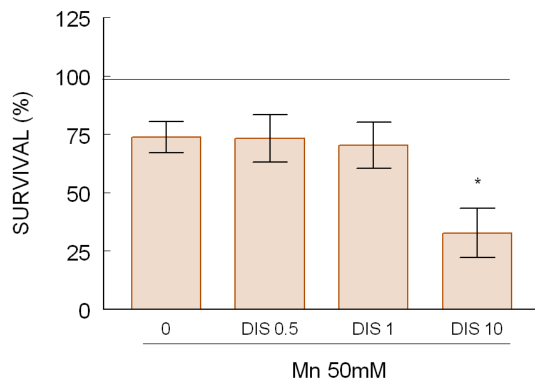


Figura 3

A)



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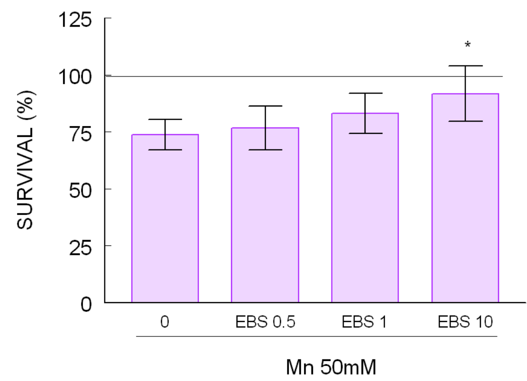


C)

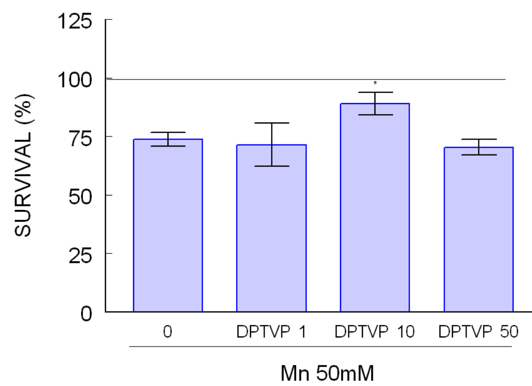


Figura 4

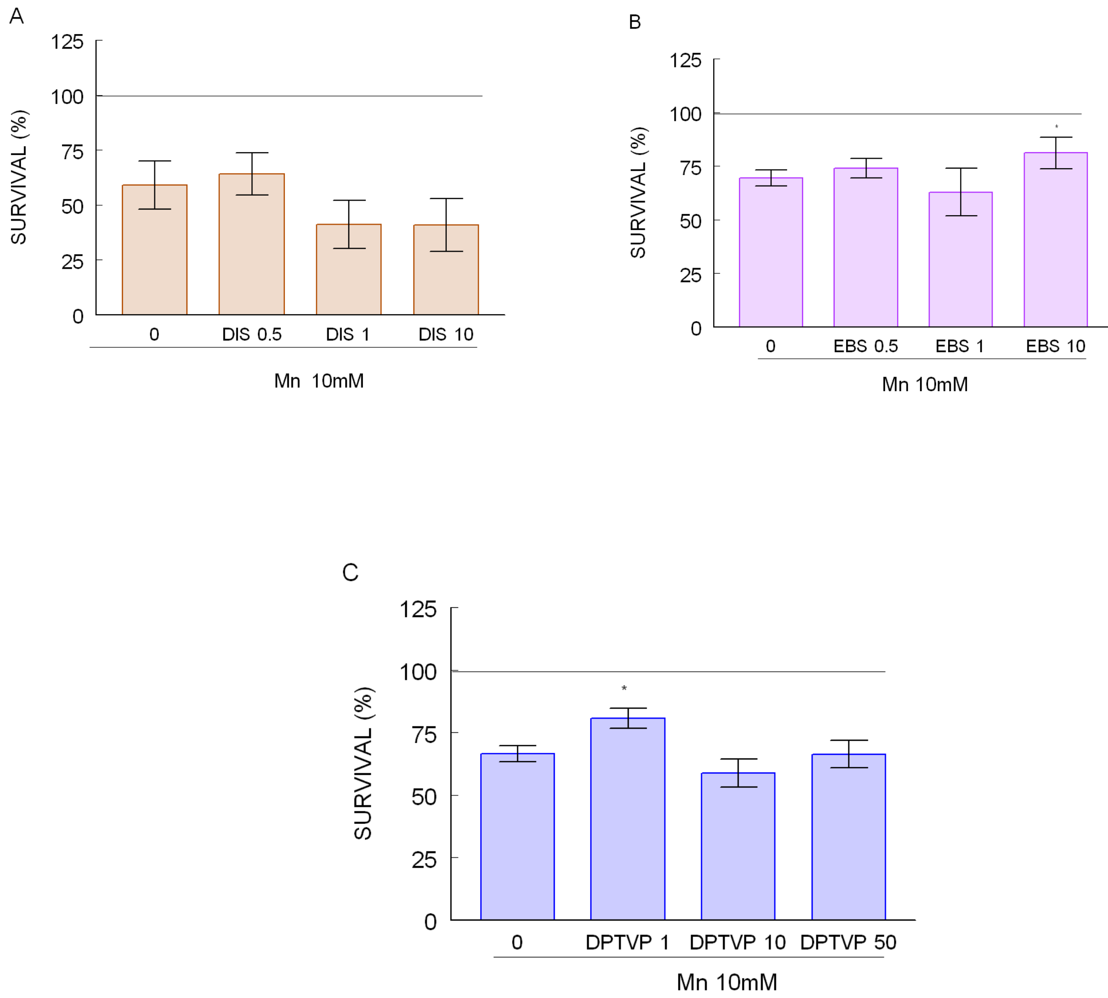
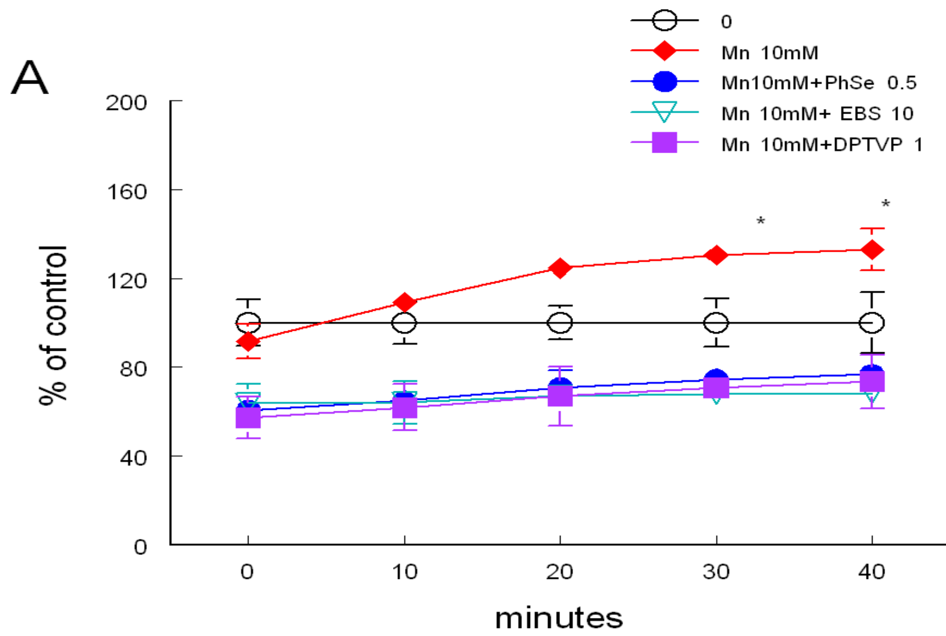


Figura 5



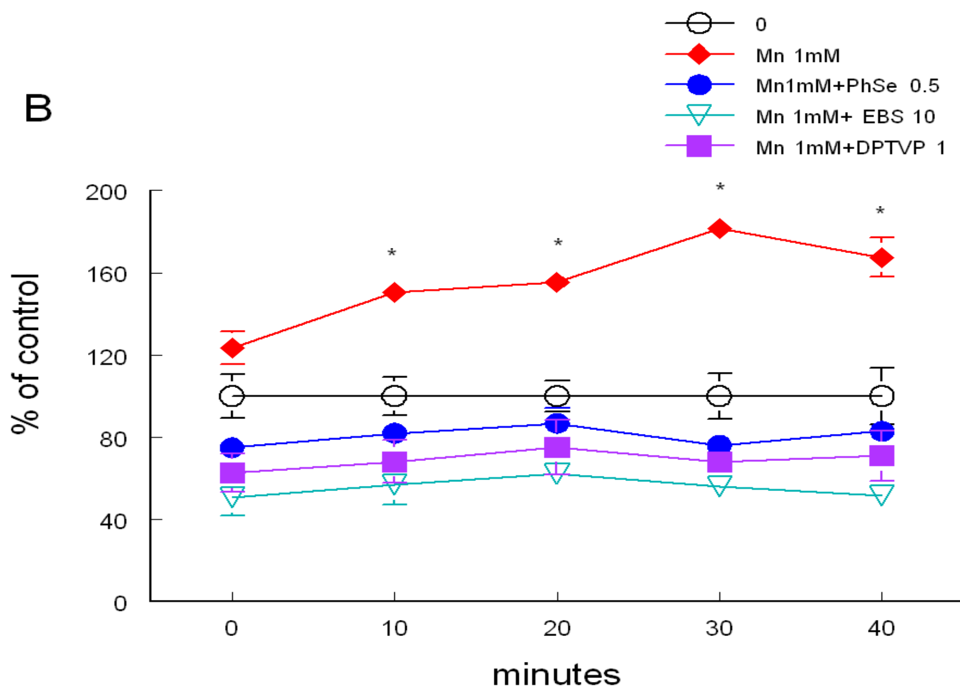
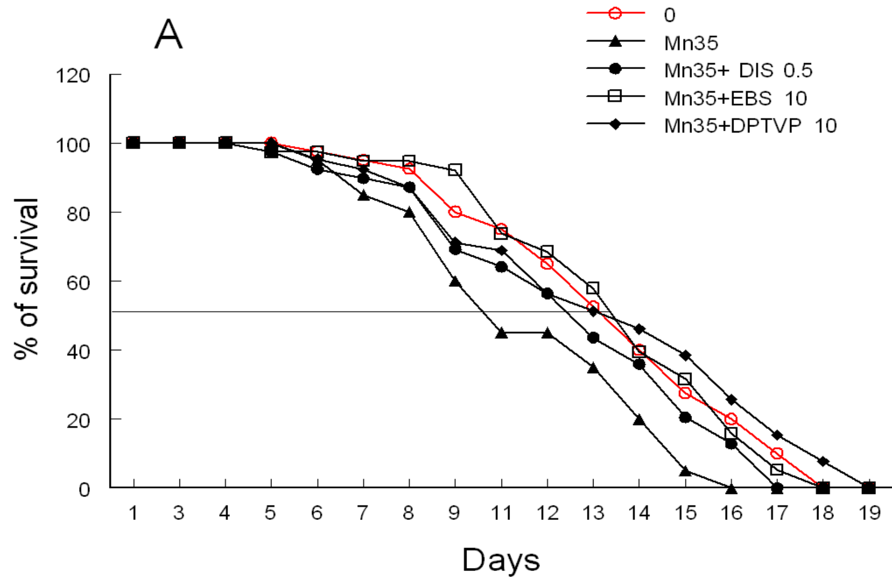


Figura 6



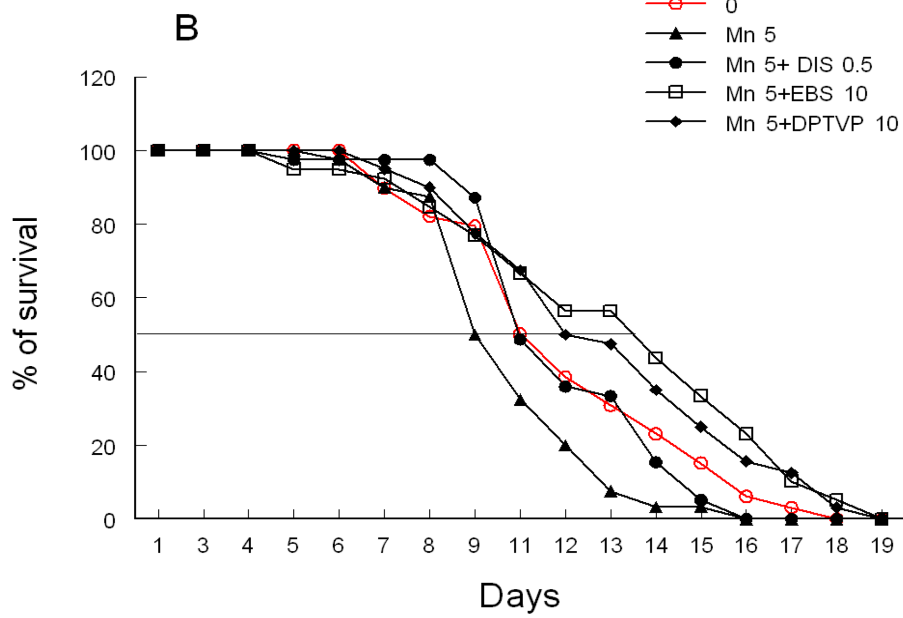
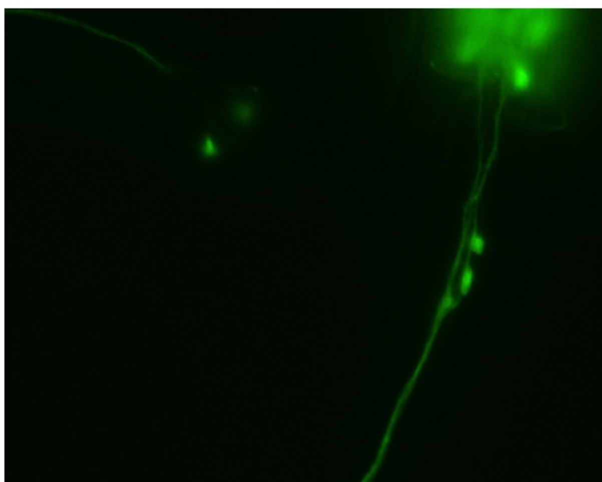


Figura 7

A



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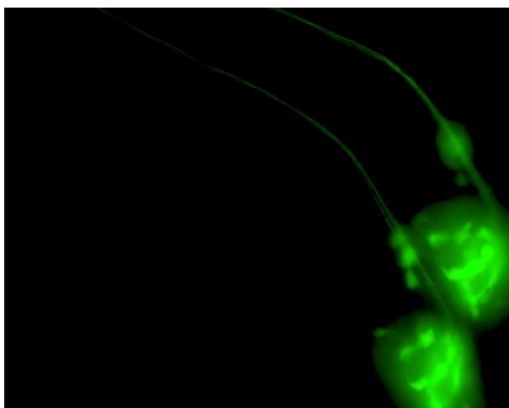

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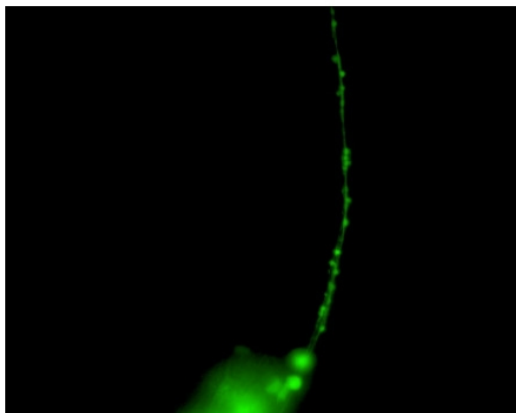
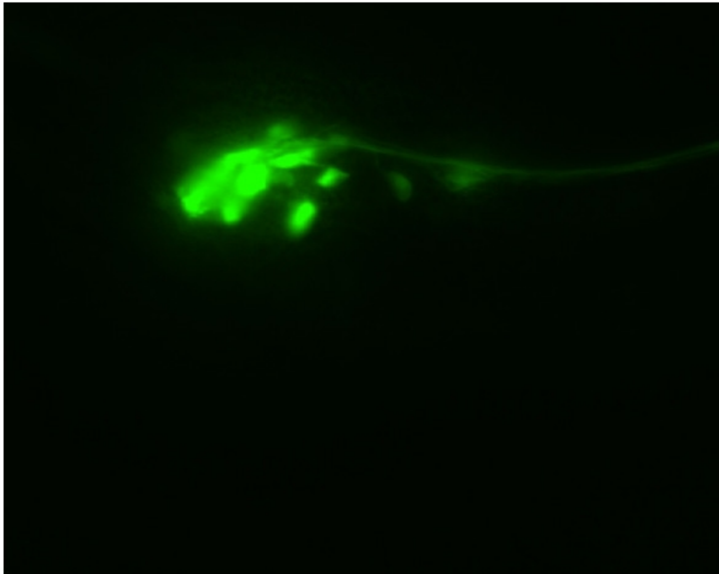


Figura 8

A



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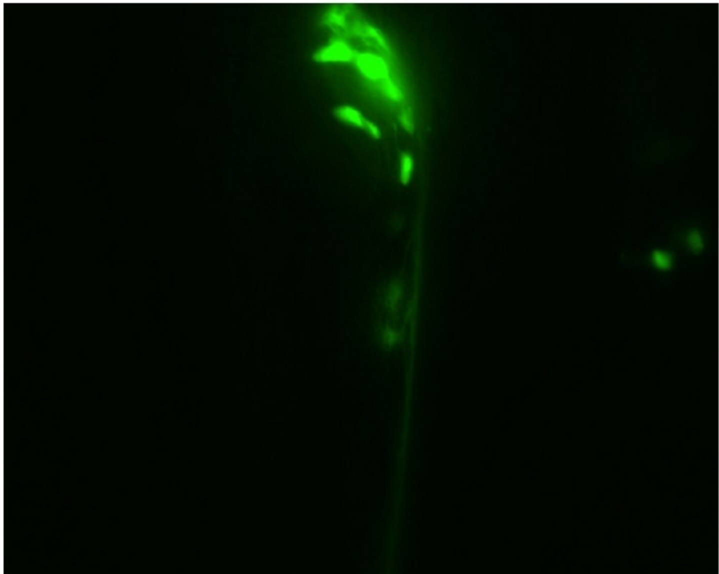

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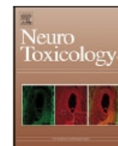

Involvement of striatal lipid peroxidation and inhibition of calcium influx into brain slices in neurobehavioral alterations in a rat model of short-term oral exposure to manganese

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ABSTRACT

Manganese is an essential element for biological systems, nevertheless occupational exposure to high levels of Mn can lead to neurodegenerative disorder, characterized by excessive Mn accumulation, especially in astrocytes of basal ganglia and symptoms closely resembling idiopathic Parkinson's disease (PD). The purpose of this study was to evaluate behavioral and biochemical alterations in adult rats exposed for 30 days to 10 and 25 mg/mL of $MnCl_2$ in their drinking water. $MnCl_2$ intoxicated rats showed impaired locomotor activity in comparison to control animals. Furthermore, lipid peroxidation were increased, δ-aminolevulinate dehydratase (δ-ALA-D, an enzyme sensitive to pro-oxidant situations) activity was inhibited and $^{45}Ca^{2+}$ influx into striatal slices was decreased in rats exposed to 25 mg/mL of Mn, indicating that this brain region was markedly affected by short-term Mn exposure. In contrast, Mn exposure was not associated with characteristic extrapyramidal effects and did not modify protein oxidation, suggesting that the striatal damage represents early stages of Mn-induced damage. In addition, treatment with Mn was associated with reduced body weight gain, but there were no discernible alterations in liver and kidney function. In conclusion, Mn caused increased oxidative stress and decreased $^{45}Ca^{2+}$ influx into the striatum, which are likely linked to impaired locomotor activity, but not with the occurrence of orofacial dyskinesia.

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1. Introduction

Since the initial findings that manganese (Mn) exposure can lead to an unsteady gait and muscular weakness resembling idiopathic Parkinson's disease (PD) (Couper, 1837), several cases of Mn neurotoxicity (manganism) have been reported, particularly in miners, smelters and workers in the battery and alloy industries (Emara et al., 1971; Bowler et al., 2003). Environmental sources of Mn in air, water, soil and food have also been the subject to studies on the effects of Mn exposures to human health (Nkwenkeu et al., 2002; Huang et al., 1989; Bouchard et al., 2007). Increased Mn intoxication has been reported in association with maneb-adulterated food (Ferraz et al., 1988), potassium permanganate, which is used to purify drinking water (Xu et al., 2005), and in particular methylcyclopentadienyl manganese tricarbonyl (MMT), an automobile fuel octane enhancer. Recent studies suggest that in areas where MMT is used, atmospheric levels of Mn in the environment have increased over pre-usage levels, as well as in drinking water sources (Mergler, 1999). The increased worldwide usage of MMT has generated concern over the potential lifelong consequences of exposure to Mn (Kaiser, 2003).

Mn is an essential element for normal growth, development, cellular homeostasis and brain development. Though uncommon, Mn deficiency increases the susceptibility to convulsions (Misselwitz et al., 1995). At the other spectrum, exposures to high levels of Mn are associated with severe damage to the liver, lung, reproductive and immune systems (Misselwitz et al., 1995; Cotzias, 1958). Mn preferentially targets the central nervous system (CNS), especially the basal ganglia (Aschner, 1997; Aschner et al., 2007), causing disturbances in neurotransmitter metabolism (Montes

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Abbreviations: ALA, aminolevulinic acid; ATP, adeninosine triphosphate; DA, dopamine; DNPH, 2,4-dinitrophenylhydrazine; DTT, dithiotreitol; MDA, malondialdehide; MMT, methylcyclopentadienyl manganese tricarbonyl; Mn, manganese; PD, Parkinson's disease; ROS, reactive oxygen species; S1, low speed supernatant; SH, cisteinyl residues; TBARS, thiobarbituric acid reactive substances; TP, tongue protrusion; VCM, vacuous chewing movements; δ-ALA-D, δ-aminolevulinate dehydratase.

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et al., 2001; Miele et al., 2000), generating excess free radicals and leading to neuronal death (Olanow et al., 1996). Dopaminergic (DAergic) neurons are a primary cellular target of Mn intoxication, mimicking many of the inherent features of PD (Newland, 1999).

At the cellular level, Mn interferes with energy production by (i) inhibiting respiratory chain complexes (Zhang et al., 2004; Galvani et al., 1995; Husain et al., 1976), (ii) disrupting mitochondrial ATP production and (iii) increasing the production of oxygen radicals (Boveris and Chance, 1973). Furthermore, it has been suggested that oxidation of Mn^{2+} to the trivalent species (Mn^{3+}) (Archibald and Tyree, 1987) and its efficient transport into DAergic neurons (Aschner et al., 1992, 1999) catalyzes the oxidation of dopamine (DA) to leukoaminochrome *o*-semiquinone, a reactive and unstable metabolite (Segura-Aguilar et al., 1998).

The DA oxidation triggered by Mn has been associated with oxidation of thiols groups in molecules with biological importance (Khan et al., 2005; Smythies, 1999). This oxidation may cause, for example, inhibition of the activity of some sulphydryl-containing enzymes, where –SH groups are essential for optimal activity (Lynem, 1970). δ-Aminolevulinate dehydratase (δ-ALA-D) is a thiol-containing enzyme involved in the tetrapyrol biosynthesis. Given its ubiquitous sensitivity to cellular redox status, this enzyme has been commonly used as an optimal biomarker of intoxication, including metal exposures (Barnard et al., 1977; Farina et al., 2003; Folmer et al., 2003; Gonçalves et al., 2005; Paniz et al., 2007; Luchese et al., 2007). Nevertheless, no data are available about its sensitivity to Mn.

Despite the propensity of Mn to target DAergic neurons, other neurotransmitter systems are also targeted by excessive Mn accumulation. GABAergic (γ-aminobutyric acid) and glutamatergic neurotransmission is altered by Mn, likely contributing to dysfunction in nigrostriatal and corticostriatal DAergic pathways (Takeda et al., 2003; Gwiazda et al., 2002). These changes likely reflect behavioral sequalae of Mn exposure, commonly associated with decreased open field activity (Witholt et al., 2000; Torrente et al., 2005), memory acquisition (Diaz-Véliz et al., 2004; Tran et al., 2003) and learning (Öner and Sentürk, 1995). However, most of these studies have been carried-out in rodents injected intraperitoneally or intracebroventricularly, thus failing to reproduce human occupational/environmental exposures.

On the other hand, the effect of Mn intoxication on extrapyramidal effects, characterized by "mouth movements" or jaw tremors (Salamone et al., 1998), has not been well documented in the literature, including in rodents. Of particular importance, mouth movements and facial tremors (orofacial dyskinesia) have been associated with oxidative stress in different models of neurotoxicity, mainly when the DAergic system is involved (Burger et al., 2006; Fachinetto et al., 2007; Castro et al., 2006; Bishnoi et al., 2007).

Considering the increasing concern about environmental exposure to Mn by oral route, the objective of this study was to determine whether behavioral parameters of orofacial dyskinesia are changed in rats after a short-term oral exposure to relatively low doses of Mn. Additionally, we evaluated the exploratory activity of rodents, as well as several biochemical parameters in striatum, hippocampus, liver and kidney of treated animals in order to clarify the relationship between oxidative stress, calcium influx and Mn-induced neurobehavioral alterations.

2. Materials and methods

2.1. Materials

$^{45}Ca^{2+}$ (5 mCi/mg Ca) was purchased from Amersham Biosciences. Inorganic manganese ($MnCl_2.4H_2O$, ~99% of purity) and all other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.2. Animal treatment

Adult Wistar rats from the Universidade Federal de Santa Maria's breeding colony were maintained in an air conditioned room (20–25 °C) under natural lighting conditions with water and food (Guabi-RS, Brazil) *ad libitum*. All experiments were conducted in accordance with the Guiding Principles of the committee of animal care of Universidade Federal de Santa Maria.

Fifteen rats were divided into three experimental groups and received for 30 days in their drinking water:

(1) No metal addition (control group);
(2) 10 mg/mL of $MnCl_2$ (2.8 mg of Mn);
(3) 25 mg/mL of $MnCl_2$ (6.9 mg of Mn).

Body weights were monitored on days 0, 15 and 30 of treatment and behavioral tests were performed on day 0 and at the end of the treatment. On the last day of Mn exposure, rats were decapitated and the brains, livers and kidneys removed. Brains were dissected out to isolate the hippocampus and striatum followed by homogenization for biochemical analyses. Kidneys and livers were homogenized (1:10) in 10 mM Tris–HCl buffer (pH 7.4) and then centrifuged at 4000 × *g* for 10 min at 4 °C. The supernatant (S1) was used for the analysis (see below)

2.3. Behavioral evaluations

2.3.1. Open field

Animals were individually placed at the center of the open field apparatus (45 cm × 45 cm × 30 cm, divided into nine squares). Spontaneous ambulation (number of segments crossed with the four paws) and exploratory activity (expressed as the number of rearings on the hind limbs) were recorded for 6 min (Walsh and Cummins, 1976).

2.3.2. Quantification of orofacial dyskinesia

Rats were placed individually in glass cages (20 cm × 20 cm × 19 cm) containing mirrors under the floor to allow behavioral quantification when the animal was facing away from the observer. Animals were habituated for 6 min to the cage and then the frequency of tongue protrusion (TP) and vacuous chewing movements (VCM) were observed during a 12 min period (Burger et al., 2006).

2.3.3. Thiobarbituric acid reactive substances production

Slices (0.4 mm) of hippocampus and striatum were prepared using a McIlwain chopper. Two slices from each region were homogenized in ultra-purified water, and then the TBA reagent (15% of trichloroacetic acid, 0.375% of thiobarbituric acid and 2.5%, v/v of HCl) was added. After 30 min of incubation at 95 °C, samples were centrifuged (3000 × g, 15 min) and then TBARS levels were measured at 532 nm (Ríos and Santamaría, 1991). An aliquot of the homogenate was used for protein determination. Kidney and liver TBARS determinations were measured in S1 accordingly to Ohkawa et al. (1979).

2.3.4. δ-ALA-D activity

Hepatic, renal, striatal and hippocampal δ-ALA-D activities were assayed by measuring the rate of product (porphobilinogen, PBG) formation (Farina et al., 2003). The reaction was started after a 15 min preincubation of the homogenate's supernatant, by adding aminolevulinic acid (ALA). Incubation was carried out at 37 °C for 1, 2 or 3 h for liver, kidney and brain, respectively. The reaction product was determined using modified Ehrlich's reagent at 555 nm, with a molar absorption coefficient of 6.1 × 104 M^{-1} for

the Ehrlich-porphobilinogen salt. The reaction rates were linear with respect to time of incubation and added protein for all the experimental conditions. Simultaneously, a set of tubes was assayed in the presence of 8 mM DTT to determine its efficacy in reverse offing Mn-induced δ-ALA-D inhibition (Farina et al., 2003; Perottoni et al., 2004).

2.3.5. Protein carbonylation assay

The striatum or hippocampus were homogenized 1:10 in Tris–HCl buffer (10 mM, pH 7.4) for protein carbonyls content, according to a modified method by Levine et al. (1990). Briefly, 1 mL aliquots were mixed with 0.2 mL of 2,4-dinitrophenylhydrazine (10 mM DNPH) or 0.2 mL HCl (2 M-blank). After 1 h incubation at room temperature in a dark environment, 0.5 mL of denaturing buffer (150 mM sodium phosphate buffer, pH 6.8, containing 3% of SDS), 1.5 mL of ethanol and 1.5 mL of heptane were added and mixed for 40 s by vortex agitation, and subsequently centrifuged for 15 min at 2000 × g. Next, the isolated protein in the interface ethanol:heptane was washed twice with ethyl acetate/ethanol 1:1 (v/v) and suspended in 1 mL of denaturing buffer. Each sample was measured at 370 nm in a Hitachi U-2001 spectrophotometer and compared to a corresponding blank sample. Total carbonylation was calculated using a molar extinction coefficient of 22,000 M^{-1} cm^{-1}.

2.3.6. Calcium influx

$^{45}Ca^{2+}$ influx measurements were carried out as previously described by Meotti et al. (2007), with some modifications. Different salt solutions were used in the present studies: Krebs buffer containing (in Mm):

Buffer 1 (basal condition): 127 NaCl, 1.2 Na_2HPO_4, 0.44 KH_2PO_4, 0.95 $MgCl_2$, 0.70 $CaCl_2$, 10 glucose, and 0.50 Hepes, pH 7.4 with 5.3 KCl.
Buffer 2 (stimulated condition): 127 NaCl, 1.2 Na_2HPO_4, 0.44 KH_2PO_4, 0.95 $MgCl_2$, 0.70 $CaCl_2$, 10 glucose, and 0.50 Hepes, pH 7.4 with 80 KCl.
Buffer 3: same composition as solution 1, except that $CaCl_2$ was replaced by 10 mM of $LaNO_3$ to block calcium channels and consequently the calcium influx.
Buffer 4: same composition of solution 2, except that $CaCl_2$ was replaced by 10 mM of $LaNO_3$.

Rats were killed by decapitation, the striata were dissected and cut into 400 μm slices. Slices (0.8–1.3 mg protein) from each rat were pre-incubated for 22 min at 32 °C and subsequently transferred to the incubation media (basal and high KCl buffer) in the presence of 0.3 μCi of $^{45}Ca^{2+}$. $^{45}Ca^{2+}$ influx was performed for 15 s at 32 °C. The reaction was stopped with five washes over 2 min with ice-cold buffer 3. Immediately thereafter, slices were lysed with 0.25 ml of a solution containing 0.5 M NaOH plus 0.2% SDS and maintained at 60 °C for 5 min. Small aliquots were taken for protein measurement and the lysate was used for determination of intracellular calcium content by liquid scintillation counting.

The specific calcium influx was determined by subtracting the nonspecific calcium uptake (20–30% of the total uptake) measured in conditions 3 and 4 from the total calcium influx measured in conditions 1 and 2, and corrected by protein content.

2.3.7. Protein content determination

Aliquots from brain slices and homogenates, as well as from kidney and liver homogenates were used for the measurement of the protein content, which was assessed accordingly to Lowry et al. (1951).

2.4. Statistical analysis

Statistical significance was assessed by MANOVA (for repeated measures), or by one-way or two-way ANOVA, followed by Student–Newman–Keuls (SNK) test for post hoc comparison. Results were considered statistically significant when $p < 0.05$.

3. Results

3.1. Body weight

At the end of the short-term Mn exposure, treated animals from both groups (10 and 25 mg/mL) showed a significant decrease in body weight gain compared with controls (Table 1, $p < 0.05$). When considering the exposure time, we also observed statistical difference between the control group and 25 mg/mL group ($F(4, 24) = 3.35$, $p < 0.05$; Table 1).

3.2. Behavioral alterations

Mn treatment (10 and 25 mg/mL) caused a significant decrease in the number of crossings when compared with the control group ($p < 0.05$, Fig. 1A). In contrast, rearing frequency was indistinguishable between the Mn-treated and control groups (Fig. 1B). The vacuous chewing incidence was decreased only in the group treated with high Mn levels (25 mg/mL) ($p < 0.05$, Fig. 1C), while tongue protrusion (TP) frequency was decreased in both Mn-treated groups compared with controls ($p < 0.05$, Fig. 1D).

3.3. Biochemical alterations

Hepatic and renal TBARS levels and δ-ALA-D activity were unchanged after Mn exposure (Table 2) and indistinguishable from control values.

In contrast to the liver and kidney, TBARS levels were significantly elevated in the striatum, but not in the hippocampus of 25 mg/mL Mn-treated rats ($p < 0.05$, Fig. 2A and B). Furthermore, a significant treatment x DTT interaction ($p < 0.05$, Fig. 3A) was noted for striatal δ-ALA-D activity in rats treated with 25 mg/mL Mn ($p < 0.05$, Fig. 3A). This effect was partially reversed by DTT. Hippocampal δ-ALA-D was unaffected by Mn exposure (Fig. 3B).

Table 1
Effects of the long-term exposure to Mn on the body weight and body weight gain of rats

	BW 0 (g)	BW 30 (g)	BW gain (g)
Control	225.2 ± 12.02	253.6 ± 13.2	28.4 ± 3.2
10 mg/mL	214.8 ± 10.6	228.8 ± 10.3	14.0 ± 2.8*
25 mg/mL	222.6 ± 5.5	236.8 ± 8.7	14.2 ± 3.8*

Data are expressed as mean ± S.E.M. for five animas in each group. * indicates significant difference from control group, $p < 0.05$ followed by Duncan post hoc test.

Table 2
TBARS levels and δ-ALA-D activity of kidney and liver of animals treated for 30 days without or with Mn in drinking water

	TBARS (nmol MDA/mg protein)		δ-ALA-D activity (nmol PBG/(mg protein h))	
	Liver	Kidney	Liver	Kidney
Control	2.68 ± 0.2	2.56 ± 0.2	12.5 ± 1.3	2.8 ± 0.3
10 mg/mL	3.06 ± 0.4	2.53 ± 0.5	11.7 ± 1.0	2.6 ± 0.3
25 mg/mL	2.9 ± 0.4	2.2 ± 0.2	11.2 ± 1.5	3.2 ± 1.1

Data are expressed as mean ± S.E.M. for five animas in each group.

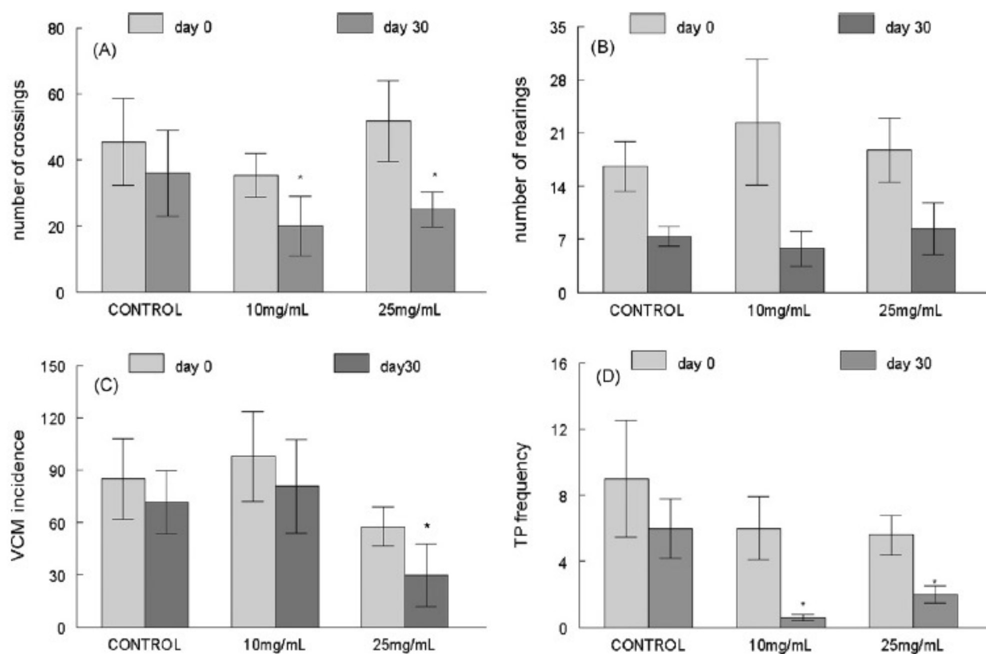

Fig. 1. Behavioral analysis of rats exposed to 30 days with 10 and 25 mg/kg of Mn in drinking water. (A) Number of crossings; (B) rearing activity; (C) number of vacuous chewing movements; (D) number of tongue protrusions. Data were analyzed by one-way ANOVA and expressed as mean ± S.E.M. (n = 5). * indicates statistical difference from control group ($p < 0.05$, SNK post hoc test).

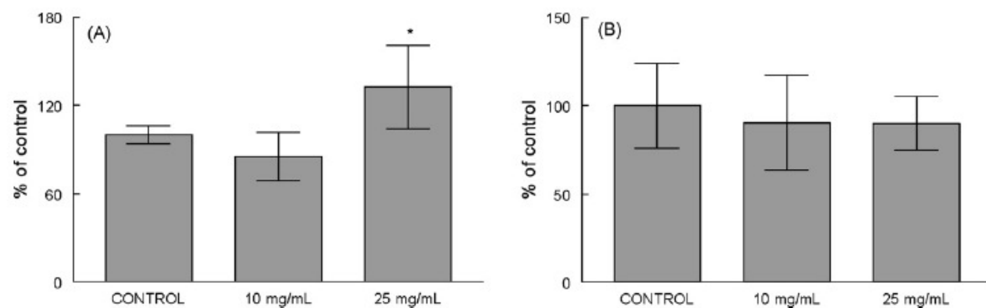

Fig. 2. TBARS levels of animals treated with Mn for 30 days in drinking water. (A) Striatum (B) hippocampus. Results are expressed as percentage of control. 100% of control corresponds to 0.448 nmol MDA/mL for striatum (A) and 0.885 nmol MDA/mL for hippocampus. Each bar represents mean ± S.E.M. (n = 5). * indicates statistical difference from control group by one-way ANOVA, following by SNK post hoc test ($p < 0.05$).

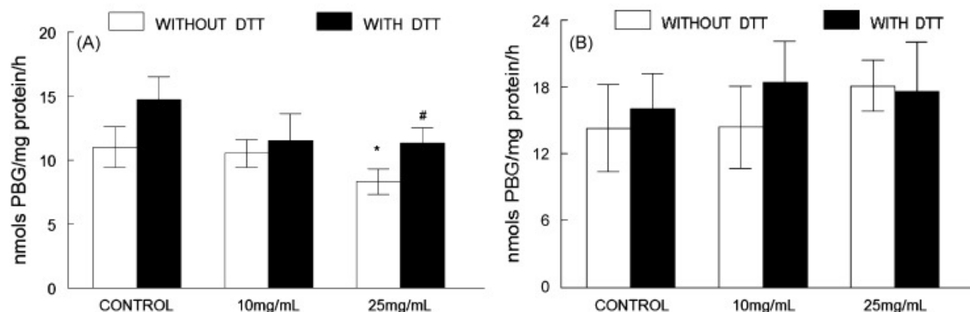

Fig. 3. δ-ALA-D activity of rats exposed to Mn. White bars represents enzyme activity in the absence of DTT, while black bars represents in the presence of DTT. (A) Striatum (B) hippocampus. Data were analyzed by one-way and two-way ANOVA and expressed as mean ± S.E.M. (n = 5). * indicates statistical difference from control group and # indicates difference from without DTT assay ($p < 0.05$, SNK post hoc test).

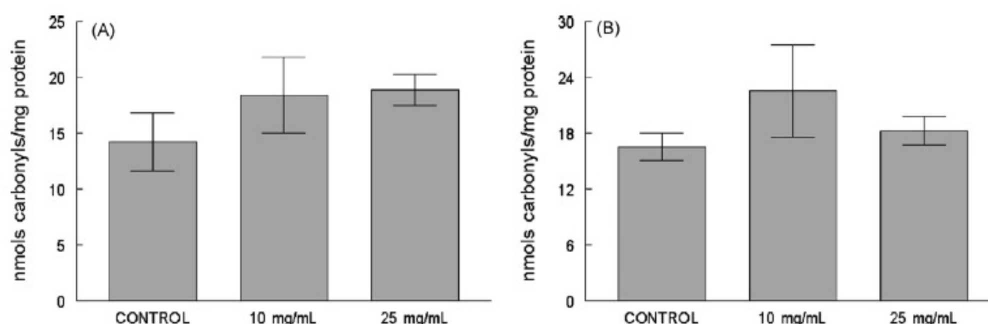

Fig. 4. Protein carbonyl content in striatum (A) and hippocampus (B) of rats after 30 days of Mn exposure. Data are expressed as mean ± S.E.M. ($n = 5$) and were analyzed by one-way ANOVA.

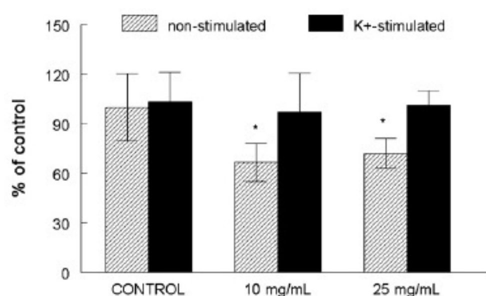

Fig. 5. $^{45}Ca^{2+}$ influx into striatal slices of rats intoxicated with Mn for 30 days in drinking water. Data were analyzed by one-way ANOVA and expressed as mean ± S.E.M. ($n = 5$). Results are expressed as percentage of control (100% corresponds to 6.56 nmol of $^{45}Ca^{2+}$/mg protein). * indicates statistical difference from control group ($p < 0.05$, SNK post hoc test).

Protein oxidation was also unchanged in both brain regions of Mn-treated rats (Fig. 4A and B) when compared with controls.

Consistent with changes in TBARS levels and δ-ALA-D activity, $^{45}Ca^{2+}$ influx was altered in the striatum. Under non-K^+-stimulated conditions, there was a significant decrease in the cation influx into striatal slices of animals treated with 10 or 25 mg/mL of Mn ($p < 0.05$, Fig. 5) compared with controls. However, under depolarizing conditions (high KCl concentration), Mn treatment did not modify calcium influx into striatal slices.

4. Discussion

Human exposure to Mn is of growing concern given its ubiquitous nature and prevalence both in the environment and occupational settings. A recent study suggests that high levels of Mn in drinking water (>300 μg/L) are associated with reduced intellectual function in establishing (Wasserman et al., 2006). In the present study, we have demonstrated that short-term oral exposure to relatively low doses of manganese impairs locomotor activity in rats, without increased orofacial dyskinesia (as measured by VCM and TP). Furthermore, this treatment caused increase oxidative stress and inhibition of $^{45}Ca^{2+}$ influx in striatum, while sparing the hippocampus. Kidney and liver were unaffected after the short-term exposure, as evidenced by unchanged δ-ALA-D activity and TBARS levels.

The PD-like effects of dopamine (DA) depleting drugs are described as slowing-down in motor activity, loss of spontaneous movement, rigidity, postural abnormalities and resting tremor (Factor and Weiner, 1988; Barbeau, 1986). Corroborating the vulnerability of DAergic neurons to Mn, we observed a significant decrease in several behavioral parameters, including open-field crossings, whereas no alterations in the number of rearings were noted.

The biochemical analyses revealed that Mn-induced oxidative stress was inherent only to the striatum of animals treated with 25 mg/mL of Mn. Notably, an association between oxidative stress in the striatum and impairment of locomotor activity has been previously described (Oliveira et al., 2007). Increased oxidative stress was absent in rats treated with 10 mg/mL, suggesting that the decrease in locomotor activity in these animals was independent of oxidative stress. The reduced locomotion likely reflects the progressive decrease in DA release caused by Mn (Guilarte et al., 2006). In addition, since ambulation (crossings) was decreased after exposure to Mn we invoke a general suppressing effect of Mn on motor activity.

Contrary to our expectations, VCM and TP (indicators of PD-like symptoms), were decreased upon Mn exposure. Of particular importance, VCM and TP have been used as indicators of tardive dyskinesia (TD) (Salamone et al., 1998). Although oxidative stress plays a central role in TD, the occurrence of VCM and TP is always associated with DAergic supersensitivity, which precedes neuronal cell death (Casey, 1995). However, in the case of Mn intoxication, DAergic supersensitivity is not apparent before DAergic neurons death, possibly because Mn is not an antagonist of DAergic receptors (Guilarte et al., 2006). Thus, these behavioral parameters are possibly better indicators of TD than Parkinson's disease.

It has been demonstrated that a main mechanism of Mn-induced neurotoxicity is via ROS generation (Aschner et al., 2007; Zhang et al., 2004; Erikson et al., 2004). One of the proposed mechanisms is by oxidation of divalent forms of Mn (Mn^{2+}) to the trivalent form (Mn^{+3}), which is significantly more reactive. Mn^{+3} is able to catalyze DA oxidation, leading to the formation of leukoaminochromes, which are exceedingly toxic to cells (Diaz-Véliz et al., 2004). On the other hand, it has been suggested that the production of Mn in the trivalent oxidation state is not of toxic significance, and that the divalent form is indeed responsible or oxidative damage (Gunter et al., 2006). In fact, it has been found that Mn^{2+} can inhibit mitochondrial respiratory chain complexes, causing decreased ATP production and leading to increased rate of production of oxygen radicals (Boveris and Chance, 1973). In the present study, we observed that lipid peroxidation was augmented in the striatum of intoxicated rats (25 mg/mL). Nevertheless, the content of oxidized proteins, measured by carbonylation, was unchanged (compared with controls) in both Mn-treated groups. This suggests that proteins are less susceptible to oxidation than lipids and that the process of free radical generation in the striatum of Mn-treated animals was in the early phases of the toxic cascade.

Furthermore, it has been postulated that Mn-induced neuronal injury is associated with suppression of ATP-dependent Ca^{2+} waves in astrocytes, altering the purinergic signalization (Tjalkens et al., 2006). Here, we observed a decrease in $^{45}Ca^{2+}$ influx into striatal slices of rats treated with 10 and 25 mg/mL of $MnCl_2$, corroborating in vitro studies with isolated neuronal cells (Tjalkens et al., 2006). Moreover, K^+-stimulated $^{45}Ca^{2+}$ uptake was unchanged by Mn, suggesting that its effect is dependent upon the non-depolarizated condition of the medium.

Interestingly, we observed such alterations only in the striatum, but not in hippocampus. This is in agreement with previous findings regarding Mn accumulation in striatum as a target tissue. It is known that once ingested, Mn is absorbed in the intestine (less than 5% in rodents, Mena, 1981; Teeguarden et al., 2007) and is distributed in brain in a non-homogeneous way (Dobson et al., 2004). Several reports have shown that Mn levels are elevated mainly in the basal ganglia region of rats after oral intake (Morello et al., 2008). Moreover, Shuckla and Chandra (1981), performing a similar experimental model to ours, demonstrated that even at a low dose (1 mg/mL), Mn levels in the striatum of treated rats were 76% increased *versus* control after 30 days of exposure in drinking water (2.21 μg of Mn/g of tissue in controls and 3.89 μg/g in Mn-treated animals). Morello et al. have found that after 13 weeks of treatment with 20 mg/mL of Mn in drinking water, striatal Mn levels were approximately 4 μg of Mn/g of tissue, while in controls they have found 1.5 μg/g. In the present study we did not measure Mn levels in the tested structures given that these previous studies already provided the necessary information for our investigation.

In the present study we also evaluated general aspects of Mn intoxication. The decrease in body weight gain at the highest dose corroborates earlier studies in different animal models (Brenneman et al., 2000; Tapin et al., 2006). However, this effect was not associated with biochemical changes in liver and kidney. δ-ALA-D activity and TBARS assay remained unchanged in both Mn-treated groups. Our work demonstrated for the first time that low doses of Mn in a short-term exposure does not alter δ-ALA-D activity in kidney, liver and also in hippocampus of animals. Nevertheless, in the striatum of rats treated with 25 mg/mL there was a significant inhibition of δ-ALA-D activity. This effect likely reflects the capacity of this brain region to preferentially accumulate Mn given the lack of effect on this enzyme's activity within the hippocampus. Furthermore, as dithiotreitol (DTT), a –SH group donor, restored the Mn-inhibited enzyme's activity, we propose that Mn directly or indirectly (by DA oxidation) oxidizes thiols groups of this enzyme. This inhibition likely is associated with decreased haemoglobin, cytochromes and catalase production, and increased concentration of its substrate, aminolevulinic acid (ALA), which possesses prooxidant action that would be, at least in a small part, responsible for the increase in the lipid peroxidation in striatum (Bechara et al., 1993).

Taken together, our results clearly demonstrate that at relatively low doses, oral exposure for only 1 month, Mn causes biochemical alterations in the striatum and in the locomotor activity in rats. Our findings establish that Mn-induced impairment in locomotor activity is, at least in part, linked to increased lipid peroxidation, inhibition of δ-ALA-D activity and decreased $^{45}Ca^{2+}$ influx in the striatum. Furthermore, our data support the hypothesis that VCM incidence and TP frequency are not ideal parameters for the detection of orofacial dyskinesia in Mn-induced PD-like symptoms in rats.

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References

Archibald FS, Tyree C. Manganese poisoning and the attack of trivalent manganese upon catecholamines. Arch Biochem Biophys 1987;256:638–50.
Aschner M, Gannon M, Kimelberg HK. Manganese uptake and efflux in cultured cell astrocytes. J Neurochem 1992;58:730–5.
Aschner M, Guilarte TR, Schneider JS, Zheng W, Manganese:. Recent advances in understanding its transport and neurotoxicity. Toxicol Appl Pharmacol 2007;221:131–47.
Aschner M, Vrana KE, Zheng W. Manganese uptake and distribution in the central nervous system (CNS). NeuroToxicology 1999;20:173–80.
Aschner M. Manganese neurotoxicity and oxidative damage. In: Connor JR, editor. Metal and oxidative damage in neurological disorders. New York: Plenum Press; 1997. p. 77–93.
Barbeau A. Parkinson's disease: clinical features and ethio pathology. In: Vinken PJ, Bruyn GN, Klawans HL, editors. Handbook of clinical neurology vol 5 (49) Extrapyramidal disorders, North Holland: Elsevier; 1986. p. 87–152.
Barnard GF, Itoh R, Hohberger LH, Shemin DJ. Mechanism of porphobilinogen synthase—possible role of essential thiol-groups. J Biol Chem 1977;252: 8965–74.
Bechara EJH, Medeiros MHG, Monteiro HP, Hermes-Lima M, Pereira B, Demasi M, et al. A free radical hypothesis of lead poisoning and in Born porphyrias associated with 5-aminolevulinic overload. Quim Nova 1993;16:.
Bishnoi M, Kumar A, Chopra K, Kullarni SK. Comparative neurochemical changes associated with chronic administration of typical ad atypical neuroleptics: implications in tardive dyskinesia. Indian J Exp Biol 2007;45:175–9.
Bouchard M, Laforest F, Candela L, Ballinger D, Mergler D. Hair manganese and hyperactive behaviours: pilot study of school-age children exposed through tap water. Environ Health Perspex 2007;115:122–7.
Boveris A, Chance B. The mitochondrial generation of hydrogen peroxide: generation properties and effect of hyper baric oxygen. Biochem J 1973;134:707–16.
Bowler MR, Givens S, Diamond E, Booty A, Hartley C, Roles HA. Neuropsychological squeal of exposure to welding fumes in a group of occupationally exposed men. Into J Hug Environ Health 2003;206:517–29.
Brenneman KA, Wong BA, Bioccelate MA, Costa ER, Gross EA, Dorman DC. Direct olfactory transport of inhaled manganese ($MnCl_2$) to the rat brain: toxic kinetic investigations in a unilateral nasal occlusion model. Toxicol Appl Pharmacol 2000;169:238–48.
Burger ME, Fachinetto ME, Wagner C, Perottoni J, Pereira RP, Zuni G, et al. Effects of biphenyl dieseline on orofacial dyskinesia model in rats. Brain Res Bull 2006;70:165–70.
Casey DE. Tardive dyskinesia-pathophysiology. In: Bloom FE, Kupfer DJ, editors. Pyschopharmacology: the fourth generation of progress. New York: Raven Press; 1995. p. 1497–502.
Castro JPMV, Frussa-Filho R, Fukushiro DF, Silva RH, Medrano WA, Abílio VC. Possible antioxidant and neuroprotective mechanisms of zolpidem in attenuating typical and anti-psychotic-induced orofacial dyskinesia—a biochemical and neurochemical study.. Brain Res Bull 2006;68:436–41.
Cotzias GC. Manganese in health and disease. Physiol Rev 1958;38:503–32.
Couper J. On the effect of black oxide of manganese when inhaled in the lungs. Br Ann Med Pharmacol 1837;1:41–2.
Diaz-Vêliz G, Mora S, Goméz P, Dossi MA, Montiel J, Arriagada C, et al. Behavioral effects of manganese injected in the rat substantia nigra ate potentiated by dicumarol, a DT-diaphorase inhibitor. Pharmacol Biochem Behav 2004;77:245–51.
Dobson AW, Erikson KM, Aschner M. Manganese neurotoxicity. Ann N Y Acad Sci 2004;1012:115–28.
Emara AM, El-Ghawabi SM, Madkour OI, El-Samra GH. Chronic manganese poisoning in the dry battery industry. Br J Ind Med 1971;28:78–82.
Erikson KM, Dobson AW, Dorman DC, Aschner M. Manganese exposure and induced oxidative stress in the rat brain. Sci Total Environ 2004;334:409–16.
Fachinetto R, Vilarinho JG, Pereira RP, Wagner C, Puntel R, Avila DS, et al. Valleriana oficinalis does not alter the orofacial dyskinesia induced by haloperidol in rats: role of opamine transporter. Progr Neuro-Psychopharmacol Biol Psyc 2007;31:1478–86.
Factor SA, Weiner WJ. The current clinical picture of Parkinson's disease. In: Hefty F, Weiner WJ, editors. Neurology. New York: Plenum Press; 1988. p. 1–9.
Farina M, Brandão R, Lara FS, Soares FAA, Souza DO, Rocha JBT. Mechanisms of the inhibitory effects of selenium and mercury on the activity of δ-aminolevulinate dehydratase from mouse liver, kidney and brain. Toxicol Lett 2003;139:179–87.
Ferraz HB, Bertolucci PH, Pereira JS, Lima JG, Andrade LA. Chronic exposure to the fungicide maneb may produce symptoms and signs of CNS manganese intoxication. Neurology 1988;38:550–3.
Folmer V, Soares JCM, Gabriel D, Rocha JBT. A high fat diet inhibits delta-aminolevulinate dehydratase and increases lipid peroxidation in mice (*Mus musculus*). J Nut 2003;133:2165–70.
Galvani P, Fumagalli P, Santagostino A. Vulnerability of mitochondrial complex in PC12 cells exposed to manganese. Eur J Pharmacol 1995;293:377–83.

Gonçalves TL, Erthal F, Corte CLD, Muller LG, Piovezan CM, Nogueira CW, et al. Involvement of oxidative stress in the pre-malignant and malignant states of cervical cancer in women. Clin Biochem 2005;38:1071–5.

Guilarte TR, Chen MK, McGlothan JL, Verina T, Wong DF, Zhou Y, et al. Nigrostriatal dopamine system dysfunction and subtle motor deficits in manganese-exposed non-human primates. Exp Neurol 2006;202:381–90.

Gunter TE, Gavin CE, Aschner M, Gunter KK. Speciation of manganese in cells and mitochondria: a search for the proximal cause for manganese neurotoxicity. Neurotoxicology 2006;27:765–76.

Gwiazda RH, Lee D, Sheridan J, Smith DR. Low cumulative manganese exposure affects striatal GABA but not dopamine. Neurotoxicology 2002;23:69–76.

Huang CC, Chu NS, Lu CS, Wang JD, Tsai JL, Tzeng JL, et al. Chronic manganese intoxication. Arch Neurol 1989;46:1104–6.

Husain R, Seth PK, Chandra SV. Early inhibition of succinic dehydrogenase by manganese in rat gonads. Bull Environ Contam Toxicol 1976;16:118–21.

Kaiser J. Manganese: a high-octane dispute: a debate over the health effects of airborne manganese is heating up as more and more countries begin adding the metal to gasoline (News).. Science 2003;300:926.

Khan FH, Sen T, Maiti AK, Jana S, Chatterjee U, Chakrabarti S. Inhibition of rat brain mitochondrial electron transport chain activity by dopamine oxidation products during extended in vitro incubation: implications for Parkinson's disease. Biochem Biophys Acta 2005;1741:65–74.

Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, et al. Determination of carbonyl content in oxidatively modified proteins. Meth Enzymol 1990;86:464–78.

Lowry OH, Rosenbrough NJ, Lewis-Farr A, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.

Luchese C, Zuni G, Rocha JBT, Nogueira CW, Santos FW. Cadmium inhibits δ-aminolevulinate dehydratase from rat lung in vitro: interaction with chelants and antioxidants agents. Chem Biol Interact 2007;165:127–37.

Lynem F. Functional sulphydryl groups in enzymic catalysis. Biochem Soc Symp 1970;31:1–19.

Mena I. Manganese. In: Bronner F, Cobrum JW, editors. Disorders on mineral metabolism. New York: Academic press; 1981. p. 233–70.

Meotti FC, Fachinetto R, Maffi LC, Missau FC, Pizzolatti MG, Rocha JBT, et al. Antinociceptive action of myricitrin: involvement on the K^+ and Ca^{2+} channels. Eur J Pharmacol 2007;567:198–205.

Mergler JD. Manganese: at what level can delirious effects occur? Can J Neurol Sci 1999;23:93–4.

Miele M, Serra PA, Esposito G, Delogu MR, Migheli R, Rocchitta G, et al. Glutamate and catabolites of high energy phosphates in the striatum and brainstem of young and aged rats subchronically exposed to manganese. Aging 2000;12:393–7.

Misselwitz B, Muhler A, Weinmann HJ. A toxicological risk for using manganese complexes? A literature survey of existing data through several medical specialties Investig Radiol 1995;30:611–20.

Montes S, Alcaraz-Zubeldia M, Muriel P, Rios C. Striatal manganese accumulation induces changes in dopamine metabolism in the cirrhotic rat. Brain Res 2001;891: 123–9.

Morello M, Canini A, Mattioli P, Sorge RP, Alimonti A, Bocca B, et al. Sub-cellular localization of manganese in the basal ganglia of a normal and manganese-treated rats: an electron-spectroscopy imaging and electron energy-loss spectroscopy study. NeuroToxicology 2008;29:60–72.

Newland MC. Animal models of manganese's neurotoxicity. Neurotoxicology 1999;20: 415–32.

Nkwenkeu SF, Kennedy G, Philippe S, Zayed J. Oral manganese intake estimated with dietary records and with direct chemical analysis. Sci Total Environ 2002;287:147–53.

Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric reaction. Anal Biochem 1979;95:351–8.

Olanow CW, Good PF, Shinotoh H, Hewit KA, Vingerhoets F, Snow BJ, et al. Manganese intoxication in the rhesus monkey: a clinical, imaging, pathologic and biochemical study. Neurology 1996;46:492–508.

Oliveira MR, Pasquali MAB, Silvestrin RB, Souza TM, Moreira JCF. Vitamin A supplementation induces a prooxidative state in the striatum and impairs locomotory and exploratory activity of adult rats. Brain Res 2007;1169:112–9.

Öner G, Sentürk ÜK. Reversibility of manganese-induced learning defects in rats. Food Chem Toxicol 1995;33:559–63.

Paniz C, Bairros A, Valentini J, Charao M, Bulcão R, Moro A, et al. The influence of the serum vitamin C levels on oxidative stress biomarkers in elderly women. Clin Biochem 2007;40:1367–72.

Perottoni J, Rodrigues OED, Paixão MW, Zuni G, Lobato LP, Braga AL, et al. Renal and hepatic ALA-D activity and selected oxidative stress parameters of rats exposed to inorganic mercury and organoselenium compounds. Food Chem Toxicol 2004;42:17–28.

Ríos C, Santamaría A. Quinolinic acid is a potent lipid peroxidant in rat brain homogenates. Neurochem Res 1991;16:1139–43.

Salamone JD, Mayorga AJ, Trevitt JD, Cousins MS, Conlan A, Nawab A. Tremulous jaw movements in rats: a model of parkisonian tremor. Progr Neurobiol 1998;66:591–611.

Segura-Aguilar J, Metodieva D, Welch CJ. Metabolic activation of dopamine *o*-quinones to *o*-semiquinones by NADPH reductase cytochrome P450 reductase may play an important role in oxidative stress and apoptotic effects. Biochem Biophys Acta 1998;1381:1–6.

Shuckla GS, Chandra SV. Striatal dopamine turnover and L-DOPA treatment after short-term exposure of rats to manganese. Arch Toxicol 1981;47:191–6.

Smythies J. Redox mechanisms at the glutamate synapse and their significance: a review. Eur J Pharmacol 1999;370:1–7.

Takeda A, Sotogaku N, Oku N. Influence of manganese on the release of neurotransmitters in rat striatum. Brain Res 2003;965:279–82.

Tapin D, Kennedy G, Lambert J, Zayed J. Bioaccumulation and locomotor effects of manganese sulphate in Sprague–Dawley rats following subchronic (90 days) inhalation exposure. Toxicol Appl Pharmacol 2006;211:166–74.

Tjalkens RB, Zoran MJ, Mohl B, Barhoumi R. Manganese suppresses ATP-dependent intercellular calcium waves in astrocytes networks through alteration of mitochondrial and endoplasmic reticulum calcium dynamics. Brain Res 2006;1113:210–9.

Teeguarden JG, Gearhart J, Clewell HJ III, Covington TR, Nong A, Anderson ME. Pharmacokinetic modeling of manganese. III. Physiological approaches accounting for background and tracer kinetics. J Toxicol Environ Health 2007;70:1515–26.

Torrente M, Colomina MT, Domingo JL. Behavioral effects of adult rats concurrently exposed to high doses of oral manganese and restraint stress. Toxicology 2005;211:59–69.

Tran TT, Chowanadisai W, Lönnerdal B, Le L, Parker M, Chicz-Demet A, et al. Effects of neonatal dietary manganese exposure on brain dopamine levels and neurocognitive functions. Neurotoxicology 2003;23:645–51.

Walsh RN, Cummins RA. The open-field test: a critical review. Psychol Bull 1976;83:482–504.

Wasserman GA, Liu X, Parvez F, Ahsan H, Levy D, Factor-Litvak P, et al. Water manganese exposure and children's intellectual function in Araihazar, Bangladesh. Environ Health Perspex 2006;114:124–9.

Witholt R, Gwiazda RH, Smith DR. The neurobehavioral effects of subchronic manganese exposure in the presence and absence of pre-parkisonism. Neurotoxicol Teratol 2000;22:851–61.

Xu XR, Li HB, Wang WH, Gu JD. Decolorization of dyes and textile wastewater by potassium permanganate. Chemosphere 2005;59:893–8.

Zhang S, Fu J, Zhou Z. In vitro effect of manganese chloride exposure on reactive oxygen species generation and respiratory chain complexes activities of mitochondria isolated from rat brain. Toxicol in vitro 2004;18:71–7.