UFSM

Dissertação de Mestrado

ANÁLISE DAS PROPRIEDADES ANTIOXIDANTES DAS OXIMAS 3-(FENIL HIDRAZONA) BUTANO-2-ONA E BUTANO-2,3-DIONATIOSEMICARBAZONA

Gustavo Orione Puntel

Santa Maria, RS, Brasil

2008

ANÁLISE DAS PROPRIEDADES ANTIOXIDANTES DAS OXIMAS 3-(FENIL HIDRAZONA) BUTANO-2-ONA E BUTANO-2,3-DIONATIOSEMICARBAZONA

por

Gustavo Orione Puntel

Dissertação apresentada ao Programa de Pós-Graduação em Bioquímica Toxicológica, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Mestre em Bioquímica Toxicológica**.

Santa Maria, RS, Brasil

2008

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

A comissão Examinadora, abaixo assinada,
Aprova a Dissertação de Mestrado

ANÁLISE DAS PROPRIEDADES ANTIOXIDANTES DAS OXIMAS 3-(FENIL HIDRAZONA) BUTANO-2-ONA E BUTANO-2,3-DIONATIOSEMICARBAZONA

elaborada por
Gustavo Orione Puntel

como requisito parcial para obtenção do grau de
Mestre em Bioquímica toxicológica

COMISSÃO EXAMINADORA:

.....
Prf. Dr. Félix Alexandre Antunes Soares
(Presidente/Orientador)
(UFSM)

.....
Profa. Dra. Maria Rosa Chitolina Shettinger
(UFSM)

.....
Profa. Dr. Nilda Berenice de Vargas Barbosa
(UNIPAMPA)

Santa Maria, 12 de maio de 2008

AGRADECIMENTOS

Agradeço, primeiramente, a Deus por estar sempre guiando meu caminho

Aos meus pais pelo amor, pelo incentivo, pela dedicação, pela compreensão, pelos valores, enfim por serem um ponto de referência em minha vida, um exemplo a ser seguido.

Aos meus irmãos pelo amor, por estarem ao meu lado durante toda a minha vida contribuindo diretamente em todas as minhas conquistas, que enfim também são suas.

Ao meu orientador Prof. Félix Soares acima de tudo pela amizade e confiança na realização de meus trabalhos. Agradeço pela orientação e ensinamentos despendidos em todos os momentos, pela disponibilidade de tempo, e também pelos "puxões de orelha" nos momentos certos. Além de minha gratidão, admiro-o por seu caráter e sua sabedoria.

Ao Prof. João Batista Teixeira da Rocha pela orientação e ensinamentos transmitidos desde os meus primeiros momentos em seu laboratório, até o presente. Agradeço pela prestatividade em todos os momentos em que precisei de seus conhecimentos. Além de minha gratidão, admiro-o por seu caráter e sua sabedoria.

Ao Prof. Gilson Zeni e a Prof[a]. Cristina Wayne Nogueira, o GZ e a Cris, pelo exemplo de conhecimento, de atitudes, e de competência. Agradeço pela prestatividade em todos os momentos em que precisei de seus conhecimentos. Além de minha gratidão, admiro-os por seu caráter e sua sabedoria.

Ao Prof. Vanderlei Folmer e amiga Verônica Bidinotto Britto, a "Vê". Além de colegas de profissão foram os responsáveis por fazer com que a bioquímica se tornasse algo fundamental em minha vida. Agradeço em especial a "Vê" pelos ensinamentos durante meus "primeiros passos" no laboratório.

Aos colegas do laboratório do "Tio João", desde o momento em que entrei no laboratório até o presente. Agradeço em especial aos "resistentes" Robson, Daniel, Rose, Cris

e Carol, que continuam compartilhando sua amizade, companheirismo e conhecimentos desde os meus primeiros momentos na bioquímica.

Aos Prof. Julio Soares e Prof. Juliano Perottoni pela amizade, companheirismo e conhecimentos compartilhados.

Aos colegas do Laboratório da Prof.ª Cristina e do Prof. Gilson pela amizade e companheirismo.

Aos colegas do laboratório do Prof. Félix, por agüentarem os meus "excessos" de mau-humor e de brincadeiras "sem graça". Agradeço-os pela amizade, auxílio, compreensão, conhecimento e companheirismo compartilhados diariamente. Agradeço a todos por darem sentido a minha vida acadêmica. Agradeço em especial a minha "mana" Priscila pela ajuda direta em todos os momentos da realização deste trabalho.

Aos colegas de laboratório e de graduação que tomaram outros rumos, pelo companheirismo e amizade.

Aos professores e aos colegas do Programa de Pós-Graduação em Bioquímica Toxicológica, que de alguma maneira contribuíram para a minha formação científica.

Aos funcionários Angélica, Márcia e Rinaldo pela dedicação e competência com que realizam os seus trabalhos.

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

À CAPES pela bolsa de estudos e pelos recursos financeiros concedidos.

Enfim, agradeço a todos que de alguma forma contribuíram para a realização deste trabalho.

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Bioquímica Toxicológica
Universidade Federal de Santa Maria, RS, Brasil

ANÁLISE DAS PROPRIEDADES ANTIOXIDANTES DAS OXIMAS 3-(FENIL HIDRAZONA) BUTANO-2-ONA E BUTANO-2,3-DIONATIOSEMICARBAZONA

AUTOR: Gustavo Orione Puntel
ORIENTADOR: Félix Alexandre Antunes Soares
CO-ORIENTADOR: João Batista Teixeira da Rocha
LOCAL E DATA DA DEFESA: Santa Maria, 12 de maio de 2008.

As oximas são compostos químicos utilizados para reativar a enzima actilcolinesterase (AChE) inibida por organofosforados (OPs). Os OPs, além de serem classicamente reconhecidos como inibidores da AChE, também estão envolvidos em situações que geram estresse oxidativo. Contudo, pesquisas enfocando as possíveis propriedades antioxidantes das oximas são escassas na literatura. O objetivo deste estudo foi investigar o potencial antioxidante e as propriedades tóxicas das oximas 3-(fenil hidrazona) butano-2-ona e butana-2,3-dionatiosemicarbazona em camundongos, e entender o(s) possível(is) mecanismo(s) pelo(s) qual(is) elas atuam. Inicialmente investigamos a existência, e o(s) mecanismo(s) pelo(s) qual(is) a oxima 3-(fenil hidrazona) butano-2-ona exerce suas propriedades antioxidantes (**Manuscrito 1**). Os resultados obtidos mostram que a peroxidação lipídica induzida por peróxido de hidrogênio (H_2O_2), por malonato, e por íons ferrosos (Fe^{2+}) foi diminuída em baixas concentrações da oxima. O tratamento dos camundongos com a oxima não alterou os níveis basais de peroxidação lipídica, nem preveniu a peroxidação lipídica induzida em experimentos *ex vivo*. Os resultados obtidos sugerem que a oxima 3-(fenil hidrazona) butano-2-ona pode ser empregada como um satisfatório composto antioxidante. A ausência de sinais de toxicidade após a administração *in vivo* da oxima 3-(fenil hidrazona) butano-2-ona em camundongos indica que esta pode ser uma droga segura para futuros estudos. O outro objetivo deste estudo foi investigar a existência e o(s) mecanismo(s) pelo(s) qual(is) a oxima butana-2,3-dionatiosemicarbazona exerce suas propriedades antioxidantes (**Manuscrito 2**). Os resultados obtidos indicam uma significativa atividade da oxima em neutralizar H_2O_2, o radical 1,1-difenil-2-picrilhidrazil ($DPPH^{\bullet}$), e a formação de óxido nítrico (NO), em baixas concentrações. Além disso, a oxima butana-2,3-dionatiosemicarbazona diminuiu significativamente a degradação da desoxirribose induzida por Fe^{2+} e pela reação $Fe^{2+} + H_2O_2$, e também a hidroxilação do benzoato induzida pela reação íons férricos (Fe^{3+}) + H_2O_2. Além disso, a oxima apresentou um efeito inibitório significativo na reação da σ-fenantrolina com Fe^{2+} Uma diminuição significativa na peroxidação lipídica basal e na peroxidação lipídica induzida por agentes pro-oxidantes no cérebro, fígado e rim de camundongos foi observada tanto *in vitro* quanto *ex vivo*. A oxima butana-2,3-dionatiosemicarbazona não determinou mudanças nos níveis de tióis (-SH) no fígado, rim e cérebro, bem como não modificou a atividade da enzima delta-aminolevulinato desidratase (δ-ALA-D) nestes tecidos de camundongos em experimentos *ex vivo*. Os resultados obtidos indicam que as oximas testadas apresentaram propriedades antioxidantes significativas, e que futuros estudos são necessários para aumentar o conhecimento a respeito do exato mecanismo de ação antioxidante destas oximas.

Palavras-chave: oxima 3-(fenil hidrazona) butano-2-ona; oxima butana-2,3-dionatiosemicarbazona, antioxidante, espécies reativas.

ABSTRACT

Dissertation of Master's Degree
Post-Graduate Course in Toxicological Biochemistry
Federal University of Santa Maria, RS, Brazil

ANALYSIS OF THE ANTIOXIDANT PROPERTIES OF THE 3-(PHENYL HYDRAZONE) BUTANE-2-ONE AND BUTANE-2,3-DIONETHIOSEMICARBAZONE OXIMES

AUTHOR: Gustavo Orione Puntel
ADVISOR: Félix Alexandre Antunes Soares
CO-ADVISOR: João Batista Teixeira da Rocha
DATE AND PLACE OF THE DEFENSE: Santa Maria, 12th may 2008

Oximes are chemical compounds used to reactivate the inhibited acetylcholinesterase (AChE) enzyme by organophosphates (OPs). The OPs, besides classically recognized as AChE irreversible inhibitors, are also involved in the generation of oxidative stress conditions. However, researches focusing on the possible antioxidant properties of oximes are lacking in the literature. The aim of this study was to investigate the potential antioxidant and toxic properties of 3-(phenylhydrazono)butan-2-one oxime and butane-2,3-dionethiosemicarbazone oxime in mice, and to understand the mechanism(s) by which they act. Firstly, we investigated the existence and the mechanism(s) by which 3-(phenylhydrazono)butan-2-one oxime exert its antioxidant properties (**Manuscript 1**). The obtained results show that *in vitro* hydrogen peroxide (H_2O_2), malonate, or ferrous ions (Fe^{2+})-induced lipid peroxidation was decreased by low concentrations of the oxime. Oxime treatment did not modify the basal peroxidation level nor prevented the induced lipid peroxidation determined *ex vivo*. The obtained results suggest that 3-(phenylhydrazono)butan-2-one oxime could be employed as a satisfactory antioxidant compound. The absence of toxicity signs after *in vivo* administration of 3-(phenylhydrazono)butan-2-one oxime to mice may indicate that it could be a safe drug for further studies. The other objective of this study was to investigate the existence and the mechanism(s) by which butane-2,3-dionethiosemicarbazone oxime exert its antioxidant properties (**Manuscript 2**). The obtained results indicate a significant H_2O_2, nitric oxide (NO) and 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) scavenging activity at low oxime concentrations. Besides, the butane-2,3-dionethiosemicarbazone oxime significantly diminished the deoxyribose degradation induced by Fe^{2+} or $Fe^{2+} + H_2O_2$, and also the benzoate hydroxylation induced by ferric ions (Fe^{3+}) + H_2O_2 reaction. Besides, the oxime showed a significant inhibitory effect on σ-phenantroline reaction with Fe^{2+}. A significant decrease in the basal and pro-oxidants induced lipid peroxidation in brain, liver, and kidney of mice was observed both *in vitro* and *ex vivo*. In addition, in our *ex vivo* experiments the butane-2,3-dionethiosemicarbazone oxime did not determine significant changes in thiol (-SH) levels of liver, kidney and brain, as well as did not modify the delta-aminolevulinate dehydratase (δ-ALA-D) activity in these tissues of mice. The obtained results indicate that the oximes tested depicted significant antioxidant properties, and that further studies are necessary to improve our knowledge regarding the exact antioxidant action mechanism of these oximes**.**

Key-words: 3-(phenylhydrazono)butan-2-one oxime, butane-2,3-dionethiosemicarbazone oxime, antioxidant, reactive species.

LISTA DE FIGURAS

3. Manuscritos

3.1. Manuscrito 1

3.2. Manuscrito 2

LISTA DE TABELAS

3. Manuscritos

3.2 Manuscrito 2

LISTA DE ABREVIATURAS

AChE –Acetilcolinesterase

ATP – Adenosina Trifosfato

ATPases – Adenosina Trifosfatases

$C^{\bullet}$ - Radicais de Carbono

CAT – Catalase

ChEs – Colinesterases

DNA – Ácido Desoxirribonucléico

$DPPH^{\bullet}$ - radical 1,1-difenil-2-picrilhidrazil

ER – Espécie Reativa

ERO – Espécie Reativa de Oxigênio

Fe^{2+} - Íons Ferrosos

Fe^{3+} - Íons Férricos

$FeCl_3$ – Cloreto de Ferro

GSH – Glutationa Reduzida

GSH-Px – Glutationa Peroxidase

GSH-Rd – Glutationa Redutase

GSSG – Glutationa Oxidada

GST - Glutationa-S-Transferases

H – Átomo de Hidrogênio

H_2O_2 – Peróxido de Hidrogênio

i.m. -. Intramuscular

i.p. – Intraperitoneal

LD_{50} – Dose Letal para 50 por cento da amostra

MDA – Malondialdeído

Na^+/K^+/ATPase – Sódio/Potássio/ATPases

nm - Nanômetros

NO – Óxido Nítrico

NPSH – Tióis não-Protéicos

NPS – Nitroprussiato de Sódio

O_2 – Oxigênio

O_2^- - Ânion Superóxido

$O^-_2{}^\bullet$ - Radical Ânion Superóxido

OH –Hidroxila

OH^- - Íons Hidroxilas

$OH^\bullet$ - Radical Hidroxil

$ONOO^-$ - Peroxinitrito

OPs – Organofosforados

PAN – Paraoxonase

RLs – Radicais Livres

$RO^\bullet$ – Radical Alcoxil

$RO_2^\bullet$ - Radical Peroxil

ROOH – Hidroperóxidos

s.c. - subcutânea

SDH - Sucinato Desidrogenase

-SH – Tióis

SOD – Superóxido Dismutase

SNC – Sistema Nervoso Central

S1 – Sobrenadante após centrifugação a 4000 g por 10 min (4ºC)

TBA – Ácido Tiobarbitúrico

TBARS - Espécies Reativas ao Ácido Tiobarbitúrico

δ-ALA-D – Delta-Aminolevulinato Desidratase

SUMÁRIO

APRESENTAÇÃO

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

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

Os itens, **DISCUSSÃO E CONCLUSÕES**, no final desta dissertação, apresentam interpretações e comentários gerais sobre os manuscritos contidos neste trabalho.

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

As **REFERÊNCIAS BIBLIOGRÁFICAS** se referem somente às citações que aparecem nos itens **INTRODUÇÃO** e **DISCUSSÃO** desta dissertação.

1. INTRODUÇÃO

1.1. Oximas

As oximas são compostos químicos que obedecem a fórmula molecular geral R_1R_2CNOH. Segundo a identidade dos radicais R1 e R2 ligados ao carbono que estabelece a ligação com a porção N-OH, as oximas podem ser classificadas como aldoximas ou cetoximas. As aldoximas possuem uma cadeia lateral orgânica como um dos radicais ligados ao carbono, e um átomo de hidrogênio como sendo o segundo radical. As cetoximas, por sua vez, apresentam duas cadeias laterais orgânicas como radicais. As oximas são, em sua maioria, sólidos cristalinos com pouca solubilidade em água e derivados da condensação de uma porção hidroxilamida (NH_2OH) com um aldeído ou uma cetona por catálise ácida. O termo oximas foi primeiramente definido no século XIX e deriva da contração das palavras oxigênio e imida (**ox**igênio+**im**id**a = oxima)** (Arena, 1997).

As oximas são compostos utilizados farmacologicamente no tratamento de intoxicações por pesticidas organofosforados (OPs). Nestas condições, o protocolo de tratamento compreende a utilização de um composto antagonista de receptores muscarínicos (atropina) e no uso de oximas (Wiener e Hoffman, 2004). O mecanismo de ação das oximas envolve a sua capacidade de reativar as enzimas colinesterases (ChEs), como por exemplo a AChE, reversivelmente inibida por carbamatos ou irreversivelmente inibidas pelos pesticidas OPs (Nigg e Knaak, 2000). Estudos recentes demonstraram que a ação das oximas, muitas vezes, envolve a sua reação direta como o resíduo de fosfato ligado ao resíduo de serina no sítio ativo da AChE (Stenzel e cols., 2007). Como resultado desta reação ocorre a desfosforilação da AChE seguida da fosforilação da oxima. O grupo fosfato ligado à molécula de oxima pode ser desligado de modo espontâneo, ou em uma reação enzimática catalizada pela enzima paraoxonase (PAN) (Stenzel e cols., 2007).

Em situações de intoxicação por pesticidas OPs, além da inibição irreversível da enzima AChE, ocorre o surgimento de um estado de estresse oxidativo caracterizado pelo aumento nos níveis de indicadores de dano oxidativo (Hernández e cols., 2005; López e cols., 2007; Abdollahi e cols., 2004; Banerjee e cols., 1999; Silva e cols., 2006 e 2008; Gultekin e cols., 2000; Gupta e cols., 2001; Fortunato e cols., 2006; Verma, 2001; Noriega e cols., 2002; Dowla e cols., 1996; Panemangalore e cols., 1999). Apesar da reconhecida capacidade das

oximas em reativar a enzima AChE, não há estudos relatando a existência de propriedades antioxidantes destas moléculas. Desta forma, estudos conduzidos com o objetivo de investigar a existência, bem como o(s) mecanismo(s) pelo(s) qual(is) as oximas exercem a sua atividade antioxidante poderiam auxiliar na elaboração de protocolos de tratamento para intoxicações com pesticidas OPs.

1.2. Pesticidas organofosforados (OPs)

As oximas são geralmente utilizadas como antídotos no tratamento de intoxicações por agentes tóxicos de ação no sistema nervoso (Worek e cols., 2004). Dentre os agentes tóxicos de ação no sistema nervoso estão os pesticidas organofosforados (OPs). Os pesticidas OPs exercem seus efeitos tóxicos mediante a inibição irreversível da atividade de esterases, como por exemplo as enzimas colinesterases (ChEs) (Nigg e Knaak, 2000). Dentre as ChEs que são irreversivelmente inibidas pelos OPs está a enzima acetilcolinesterase (AChE). A enzima AChE é responsável pela degradação do neurotransmissor acetilcolína (ACh) das sinapses colinérgicas, e sua inibição determina o acúmulo de ACh no espaço sináptico (Nigg e Knaak, 2000). O acúmulo de ACh no espaço sinaptico, por sua vez, pode acarretar no desenvolvimento de uma situação de super-estimulação colinérgica tanto ao nível do sistema nervoso central (SNC) quanto ao nível das junções neuromusculares periféricas (Worek e cols., 2007). Como resultado de uma super-estimulação colinérgica ocorre uma massiva disfunção de inúmeras funções fisiológicas relacionadas à funcionalidade do sistema colinérgico e eventualmente a morte por falência respiratória (Eddleston e cols., 2006).

O mecanismo de inibição enzimática exercido pelos OPs envolve a fosforilação irreversível de um resíduo de serina presente no sitio ativo das ChE. O grupo hidroxila (OH) nucleofílico do resíduo de serina presente no sitio ativo da AChE é responsável pela ligação e conseqüente hidrólise do neurotransmissor ACh. A ligação da porção acila da ACh no OH do resíduo de serina determina a sua hidrólise em colina e acetato. Após a hidrólise da ACh ocorre a liberação da colina, sendo que a porção acila da ACh permanece ligada ao resíduo de serína. Em um último momento ocorre a hidrólise do resíduo de serina acetilado com a subseqüente liberação da porção acetato. Uma vez que os fragmentos de colina e acetato deixarem o sítio ativo da AChE uma nova molécula de ACh pode ser degradada (Nigg e Knaak, 2000).

Em situações onde há uma intoxicação por OPs ocorre a ligação covalente e irreversível do átomo de fósforo presente nas moléculas dos OPs com o OH do resíduo de serina presente no sitio ativo da enzima AChE. Como resultado observa-se a inativação da

enzima AChE determinada pela sua incapacidade de liberar o grupo fosfato do seu sítio ativo (Nigg e Knaak, 2000). É importante salientar que a enzima AChE inibida por pesticidas OPs pode sofrer um processo espontâneo de desalquilação devido a ruptura da ligação entre o grupo alquil e o átomo de oxigênio e assim resultar num processo de inativação irreversível da mesma ("aging"). Por outro lado, a inibição da enzima AChE pode ser também espontaneamente reativada mediante um processo de desfosforilação espontânea da enzima inibida. A estrutura dos pesticidas OPs é fundamental para o entendimento dos processos de inibição irreversível e de reativação espontânea da enzima AChE fosforilada (Worek e cols., 2007; Stenzel e cols., 2007).

Além dos documentados efeitos dos pesticidas OPs na função de enzimas ChE como, por exemplo, da enzima AChE, estudos recentes revelam um efeito significativo destes no desenvolvimento de uma situação de estresse oxidativo (Hernández e cols., 2005; López e cols., 2007; Abdollahi e cols., 2004). Em determinadas situações os OPs podem acarretar mudanças em rotas metabólicas intracelulares e assim determinar o surgimento de uma situação de estresse oxidativo caracterizada pelo aumento no dano oxidativo (Banerjee e cols., 1999). O dano oxidativo causado por diferentes pesticidas OPs já foi caracterizado em diferentes espécies de animais, como em camundongos (Silva e cols., 2006 e 2008) e ratos (Gultekin e cols., 2000; Gupta e cols., 2001), e também em tecidos humanos (Banerjee e cols., 1999). Neste contexto, um aumento na produção de espécies reativas ao ácido tiobarbitúrico (TBARS) em cérebro de camundongos (Fortunato e cols., 2006; Verma, 2001), e eritrócitos humanos (Gultekin e cols., 2000), bem como uma redução na atividade da enzima δ-ALA-D no fígado de ratos (Noriega e cols., 2002) e em eritrócitos humanos (Dowla e cols., 1996; Panemangalore e cols., 1999) foram relatados após intoxicação por pesticidas OPs. Apesar da existência de vários estudos relacionando a intoxicação por pesticidas OPs com o desenvolvimento de dano oxidativo tecidual, o exato mecanismo pelo qual estes estão envolvidos na gênese do estado de estresse oxidativo ainda é desconhecido (Abdollahi e cols., 2004).

1.3. Estresse oxidativo

O estado de estresse oxidativo é caracterizado pelo desequilíbrio entre a geração de espécies reativas (ERs) e a capacidade dos organismos neutralizarem-nas (Gutteridge e Halliwell, 1994). As situações de intoxicação por pesticidas OPs podem acarretar na geração de ERs acima da capacidade dos sistemas de defesa antioxidante celulares (Banerjee e cols., 1999). Nestas condições, as excessivas ERs podem reagir com uma série de biomoléculas

celulares e desencadear sua disfunção num mecanismo definido como dano oxidativo (Halliwell, 2006; Augusto e cols., 2002).

1.3.1. Espécies reativas (ERs) e radicais livres (RLs)

As células aeróbias estão continuamente produzindo ERs como parte de seu processo metabólico (Halliwell, 2006). As ERs geradas em baixas concentrações cumprem um importante papel fisiológico relacionado a manutenção da "homeostase redox" envolvida nos processos de sinalização intracelular (Droge, 2002; Pacher e cols., 2007), e também nos mecanismos de defesa orgânica contra processos infecciosos e no desenvolvimento adequado da resposta inflamatória a um dano tecidual (Halliwell, 2006). No entanto, uma exacerbada geração de ERs está relacionada a origem de disfunções orgânicas características de processos patológicos (Halliwell, 2006; Augusto e cols., 2002)

Os RLs são moléculas capazes de existência independente, por isto o termo "livre", que tem um ou mais elétrons desemparelhado em sua órbita externa (Gutteridge e Halliwell, 1994). Os RLs se caracterizam por serem moléculas altamente instáveis, por terem uma vida média muito curta medida em microssegundos, e por procurarem sua estabilidade através do pareamento de seus elétrons. A reatividade dos RLs pode ser estabelecida com moléculas radicais ou não-radicais (Halliwell, 2006). As ERs, por sua vez, são moléculas que possuem uma alta reatividade mas que não são, necessariamente, RLs capazes de existência independente (Halliwell, 2006). A reatividade das ERs pode ser estabelecida pela ação destas como agentes oxidantes ou redutores das biomoléculas circundantes, o que determina que tais moléculas não sejam classificadas simplesmente como agentes oxidantes (Halliwell, 2006). Dentre as diferentes formas de ERs existentes destacam-se as derivadas de átomos de oxigênio, as chamadas espécies reativas de oxigênio (EROs). As principais EROs vinculadas ao estresse oxidativo decorrente de situações estressantes ao organismo são o radical ânion superóxido ($O^{-}_{2}{}^{\bullet}$), o radical hidroxil ($OH^{\bullet}$), o peróxido de hidrogênio (H_2O_2), o óxido nítrico (NO) e o peroxinitrito ($ONOO^{-}$).

1.3.2. Sistemas de defesa antioxidantes

Os sistemas de defesa antioxidantes são os responsáveis pela neutralização das excessivas ERs geradas durante situações estressantes ao organismo. Estes sistemas atuam de modo a controlar os níveis de ERs no meio intracelular, desta forma, impedindo a disfunção de biomoléculas intracelulares mediadas por tais espécies. De modo geral, os sistemas de defesa antioxidantes endógenos celulares são classificados em enzimáticos (a catalase (CAT),

a superóxido dismutase (SOD), a glutationa peroxidase (GSH-Px)) ou não-enzimático (as vitaminas A, E, e C, os flavonóides, as ubiquinonas e a glutationa reduzida- GSH) (Gianni e cols., 2004).

1.3.3. Dano oxidativo

O equilíbrio entre a geração de ERs e a capacidade dos sistemas de defesa antioxidantes celulares é essencial para que se mantenha a homeostase endógena necessária às funções intracelulares. A geração excessiva de ERs ou a diminuição da capacidade dos sistemas de defesa antioxidantes pode culminar com o comprometimento de biomoléculas celulares e desencadear a sua disfunção num mecanismo definido como dano oxidativo (Halliwell, 2006; Augusto e cols., 2002). O mecanismo de dano oxidativo pode estar relacionado à reação de RLs com moléculas não radicais, e a ação de ERs como agentes redutores ou oxidantes das biomoléculas teciduais circundantes (Halliwell, 2006).

1.3.3.1. Peroxidação lipídica

Dentre as biomoléculas mais acometidas pelo dano oxidativo mediado por ERs e RLs estão as membranas celulares. As membranas celulares biológicas apresentam uma estrutura geral comum que compreende uma bicamada lipídica associada a proteínas. Os componentes lipídicos das membranas são principalmente fosfolipídeos, os quais possuem uma cabeça polar e duas caudas hidrofóbicas apolares. As caudas hidrofóbicas são compostas por ácidos graxos, os quais podem apresentar uma ou mais insaturações (Alberts e cols., 1994; Halliwell e Gutteridge, 1989). O mecanismo de dano oxidativo às membranas celulares envolve a remoção de átomos de hidrogênio (H) das ligações C-H, presentes nos ácidos graxos componentes da membrana, mediada pela ação de RLs (Halliwell, 2006). Os radicais de carbono ($C^{\bullet}$), resultantes da remoção do H, reagem rapidamente com oxigênio molecular (O_2) gerando radicais peroxil ($RO_2^{\bullet}$), os quais são reativos o bastante para promover a oxidação de outros ácidos graxos e proteínas constituintes da membrana. A propagação do dano oxidativo aos componentes lipídicos das membranas biológicas é definida como peroxidação lipídica.

O comprometimento das membranas decorrentes da peroxidação lipídica determina a perda de suas características arquitetônicas. Nestas condições, as membranas tornam-se suscetíveis a criação de verdadeiras fendas iônicas, o que favorece a entrada e saída indiscriminada de metabólitos e detritos da célula, favorecendo sua ruptura e lise com conseqüente necrose celular (Josephy, 1997; Timbrell, 2000).

1.4. Marcadores de dano oxidativo empregados no estudo

1.4.1. Espécies reativas ao ácido tiobarbitúrico (TBARS)

As espécies reativas ao ácido tiobarbitúrco (TBARS) são caracterizadas como sendo produtos finais do dano oxidativo aos lipídios de membrana durante o processo de peroxidação lipídica. Dentre os produtos finais da peroxidação lipídica de membranas ocorre a formação de molondialdeído (MDA). O MDA formado pode reagir com o ácido tiobarbitúrico (TBA) e resultar na formação de complexos coloridos TBA-MDA, os quais possuem um coeficiente de absorção máxima em 532 nanometros (nm) (Ohkawa e cols., 1979). Desta forma, a análise da formação de TBARS pode ser empregada convenientemente como um índice do comprometimento lipídico oriundo do dano oxidativo resultante de uma situação estressante ao organismo (Puntel e cols., 2007).

1.4.2. Atividade enzimática delta-aminolevulinato desidratase (δ-ALA-D)

A enzima δ-ALA-D é uma enzima de natureza sulfidrílica que pode ser inibida em diferentes situações pro-oxidantes aos organismos (Folmer e cols., 2003; Perottoni e cols., 2005). Um dos mecanismos pelo qual a atividade da enzima δ-ALA-D pode ser inibida envolve a oxidação dos grupos tiólicos pela ação de pesticidas OPs (Noriega e cols., 2002; Dowla e cols., 1996; Panemangalore e cols., 1999), e pela ação de compostos com alta afinidade por grupos tiólicos (Barbosa e cols.,1998; Maciel e cols., 2000; Farina e cols., 2001). A ausência de alterações significativas na atividade da enzima δ-ALA-D indica a preservação dos grupos tiólicos presentes no sitio ativo da enzima. Desta forma, a atividade da enzima δ-ALA-D pode ser utilizada convenientemente como um marcador de dano oxidativo em situações de estresse oxidativo (Maciel e cols., 2000).

1.4.3. Níveis de grupos sulfidrílicos (-SH)

Os grupos sulfidrílicos ou tiólicos (-SH) cumprem papéis importantes nos sistemas biológicos. Os grupos –SH são constituintes da cadeia lateral do resíduo de aminoácido essencial cisteína. O resíduo de cisteína, por sua vez, está envolvido na constituição de diversas proteínas e enzimas sendo fundamental para a funcionalidade destas. Outra importante função do resíduo de cisteína é a contrução do antioxidante glutationa (GSH). A GSH é um tripeptídeo composto pelos resíduos de aminoácidos L-cisteína, L-glutamato, e glicina que é empregado como substrato para a ação de enzimas com carácter antioxidante como a glutationa peroxidase (GSH-Px), e a catalase (CAT). Além disso, a GSH é empregada

como um substrato necessário à ação das enzimas glutationa-S-transferases (GST), as quais possuem importância fundamental nos processos de metabolização de xenobióticos (Sheehan e cols., 2001). A excessiva geração de ERs, em decorrência de situações estressantes, determina a progressiva oxidação dos grupos –SH e consequente alteração na funcionalidade de enzimas, proteínas e nos sistemas de defesa antioxidantes (Sies, 1997; Vertuani e cols., 2004). Desta forma, a análise dos níveis de grupos –SH pode ser utilizada convenientemente como um marcador de dano oxidativo em situações de estresse oxidativo

1.5. Agentes pró-oxidantes usados no estudo

1.5.1. Malonato

O malonato é um inibidor reversível da sucinato desidrogenase (SDH) (EC 1.3.99.1), uma enzima chave no metabolismo oxidativo, devido à sua semelhança estrutural com o substrato da enzima, o sucinato (Maragos e Silverstain, 1995; Schulz e cols., 1996). Assim sendo, seu acúmulo prejudica o metabolismo aeróbio.

Ao inibir a SDH o malonato acarreta em uma interrupção do transporte de elétrons pela cadeia de transporte de elétrons mitocondrial, e assim do processo fosforilação oxidativa. Como resultado da interrupção no processo de fosforilação oxidativa ocorre uma depleção nos níveis de adenosina trifosfato (ATP). Nessas condições, ocorre uma despolarização das membranas celulares devido a falência de várias ATPases, principalmente da Na^+/K^+/ATPase (Greene e cols., 1993), que mantêm a tensão e o gradiente de íons através da membrana plasmática. Desta forma, a inibição da SDH causada pelo malonato leva à disfunção mitocondrial, geração de radicais livres, excitotoxicidade secundária e apoptose (Dedeoglu e cols., 2002).

1.5.2. Ferro

O ferro é o metal de transição mais abundante nos tecidos biológicos (Welch e cols., 2002). A quantidade normal de ferro presente em um adulto saudável é estimada em 3-4 g (Emery, 1991). O ferro atua como um cofator essencial para uma grande variedade de proteínas como citocromos, proteínas contendo o grupo prostético heme, e proteínas ferro-enxofre (Fe-S) (Welch e cols., 2002). Da mesma forma, enzimas contendo ferro são fundamentais para os processos bioquímicos celulares de transporte de oxigênio, metabolismo e respiração cellular, e também na síntese de DNA (Welch e cols., 2002).

Os metais de transição estão, de modo geral, envolvidos em muitas reações bioquímicas de oxidação fundamentais. Contudo, as mesmas propriedades físicas que

permitem ao ferro atuar como um eficiente cofator protéico, permitem que este promova a oxidação deletéria de importantes biomoléculas celulares, como lipídios, proteínas e o DNA (Welch e cols., 2002).

De modo geral, o ferro pode determinar a produção de ERs por diferentes mecanismos (Braughler e cols., 1986; Minotti e Aust, 1987 e 1992):

a) Por degradar hidroperóxidos lipídicos pré-existentes (ROOH) nos tecidos, formando o radical lipídico alcoxil ($RO^{\bullet}$);

b) Por participar nas reações do tipo Fenton produzindo radicais hidroxil ($OH^{\bullet}$);

c) Por formar complexos com oxigênio, tal como os complexos Fe^{2+}-O_2-Fe^{3+}, os quais podem iniciar as reações de peroxidação lipídica (Oubidar e cols., 1996).

Além disso, dados na literatura sugerem que a razão entre íons de ferro no estado ferroso (Fe^{2+}) e no estado férrico (Fe^{3+}) é um fator fundamental na iniciação e propagação das reações de peroxidação lipídica (Braughler e cols., 1986). Assim, compostos capazes de interagir com íons ferrosos (Fe^{2+}) ou férricos (Fe^{3+}) podem ajustar indiretamente a atividade desses íons por modular diferentes razões Fe^{2+}/ Fe^{3+}.

1.5.3. Peróxido de hidrogênio (H_2O_2)

O peróxido de hidrogênio (H_2O_2) é um ácido fraco líquido de coloração azul de pequena intensidade, que não apresenta coloração quando diluído, e que é levemente mais viscoso que a água. O H_2O_2 possui propriedades oxidantes bastante intensas sendo utilizado como um potente agente descolorante, desinfetante, oxidante, e antiséptico. Devido a sua intensa capacidade oxidante, o H_2O_2 é considerado uma ER altamente reativa (Greenwood e Earnshaw, 1997).

Além disso, é importante salientar que o H_2O_2 está diretamente envolvido na gênese de $OH^{\bullet}$ mediada pela reação de Fenton. Segundo a reação de Fenton íons Fe^{2+} reagem com a espécie reativa H_2O_2 resultando na formação de $OH^{\bullet}$ e íons hidroxilas (OH^-). De acordo com seu mecanismo de dano, os $OH^{\bullet}$ podem atacar açucares como a pentose desoxirribose além de reagir com outras biomoléculas intracelulares, sejam estas radicais ou não, e transformá-las em espécies reativas ao abstraírem um elétron destas (Halliwell, 2006).

1.5.4. Nitroprussiato de sódio (NPS)

O nitroprussiato de sódio (NPS) é um composto químico com a fórmula molecular $Na_2[Fe(CN)_5NO]\cdot 2H_2O$ que é classicamente reconhecido como um doador da ER óxido

nítrico (NO) na presença de agentes redutores (Bates e cols., 1991). O NO liberado da decomposição da molécula de NPS, na presença de biomoléculas intracelulares, pode reagir com ânions superóxidos (O_2^-) e determinar a formação de peroxinitritos ($ONOO^-$). A espécie reativa $ONOO^-$ está diretamente envolvida na gênese do dano oxidativo tecidual (Villa e cols., 1994).

Além de liberar NO, ao ser degradada a molécula de NPS libera complexos de pentacianoferratos, os quais também podem estar envolvidos na origem do dano tecidual induzido por NPS (Rao e cols., 1991; Roncaroli e cols., 2005; Bates e cols., 1991).

2. OBJETIVOS

2.1. Objetivo Geral

Analisar as propriedades antioxidantes e toxicológicas, bem como estudar o(s) mecanismo(s) através do(s) qual(is), as oximas 3-(fenil hidrazona) butano-2-ona e butana-2,3-dionatiosemicarbazona exercem seus possíveis efeitos antioxidantes.

2.2. Objetivos Específicos

- Investigar a capacidade das oximas estudadas em limitar a formação de TBARS induzida por diferentes agentes pro-oxidantes em diferentes tecidos de camundongo *in vitro*.
- Investigar a capacidade das oximas estudadas em neutralizar a formação de ERs tais como $OH^{\bullet}$ e NO *in vitro*.
- Investigar a capacidade das oximas estudadas em neutralizar ERs como o H_2O_2 e o radical $DPPH^{\bullet}$ *in vitro*.
- Investigar a capacidade das oximas estudadas em interagir com íons ferrosos (Fe^{2+}).
- Analisar os efeitos do tratamento de camundongos com as oximas estudadas na peroxidação lipídica basal e a induzida por diferentes agentes pro-oxidantes em diferentes tecidos de camundongos a partir de análises *ex vivo*.
- Analisar a toxicidade *ex vivo* do tratamento de camundongos com as oximas estudadas a partir da análise da atividade da enzima δ-ALA-D e dos níveis de tióis (SH) em diferentes tecidos de camundongos.
- Avaliar a toxicidade *in vivo* das oximas estudadas, quando administradas subcutaneamente em camundongos, a partir da análise da dose letal para cinqüenta por cento da amostra (LD_{50}).

3. MANUSCRITOS

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de manuscritos, os quais se encontram aqui organizado. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios manuscritos. Os manuscritos estão dispostos na forma em que foram submetidos para publicação nas revistas científicas.

3.1. – **PROPRIEDADES ANTIOXIDANTES DA OXIMA 3-(FENIL HIDRAZONA) BUTANO-2-ONA**

Manuscrito 1: aceito para publicação

ANTIOXIDANT PROPERTIES OF OXIME 3-(PHENYLHYDRAZONO) BUTAN-2-ONE

Gustavo Orione Puntel, Priscila Gubert, Gisele Louro Peres, Leandro Bresolin, João Batista Teixeira Rocha, Maria Ester Pereira, Vanessa Santana Carratu, Félix A. Antunes Soares

Archives of Toxicology

Antioxidant properties of oxime 3-(phenylhydrazono) butan-2-one

Gustavo Orione Puntel[1], Priscila Gubert[1], Gisele Louro Peres[2], Leandro Bresolin[3], João Batista Teixeira Rocha[1], Maria Ester Pereira[1], Vanessa Santana Carratu[2], Félix A. Antunes Soares[1*]

[1]Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Campus UFSM, Santa Maria, RS, Brasil.

[2]Departamento de Química, Fundação Universidade de Rio Grande, Campus Carreiros, Rio Grande, RS, Brasil.

[3]Universidade Federal do Acre, Campus Cruzeiro do Sul, Cruzeiro do Sul, AC, Brasil.

*CORRESPONDING AUTHOR:

Félix Antunes Soares

Departamento de Química - CCNE - Universidade Federal de Santa Maria

97105-900 - Santa Maria - RS - Brazil

Phone: +55-55-3220-9522

Fax: +55-55-3220-8978

E-mail: felix_antunes_soares@yahoo.com.br

ABSTRACT

Oximes are a class of compounds normally used to reverse the acetylcholinesterase (AChE) inhibition caused by organophosphates. Conversely, researches focusing on the possible antioxidant properties of these compounds are lacking in the literature. The aim of this study was to investigate the potential antioxidant and toxic properties of 3-(phenylhydrazone) butan-2-one oxime in mice. *In vitro*, hydrogen peroxide-induced lipid peroxidation was decreased by low concentrations of the oxime (0.1 μM to 1.0 μM); ($p<0.05$). Similarly, lipoperoxidation induced by malonate and iron (Fe^{2+}) was significantly decreased by the oxime (0.4 μM to 1.0 μM) ($p<0.05$). Oxime pre-treatment did not modify the basal peroxidation level nor prevented the induced lipid peroxidation determined ex-vivo. The present results suggest that 3-(phenylhydrazone) butan-2-one oxime could be a good antioxidant compound. The absence of toxicity signs after *in vivo* administration of 3-(phenylhydrazone) butan-2-one oxime to mice may indicate that it could be a safe drug for further studies.

Keywords: oximes, antioxidant, oxygen reactive species;

INTRODUCTION

Experimental points of evidence have indicated a key role for reactive species (RS) as potential cytotoxic agents (Halliwell 2006). Most importantly, several acute and chronic pathological conditions can be associated with an increase in RS production that surpasses cellular endogenous antioxidant system capacity for their neutralization (Gutteridge and Halliwell 1994; Halliwell et al. 1999). In view of the apparent role of RS in several acute and chronic human diseases, the interest in the development of new compounds that improve the antioxidant systems is of interest (Urso and Clarkson 2003; Nogueira et al. 2004; Halliwell 2006; Puntel et al. 2007).

Some organic intoxication conditions, like the ones caused by pesticides, may induce changes in intracellular metabolism routes leading to an imbalance between the generation of oxidant molecules and the intracellular antioxidant systems (Banerjee et al. 1999). Organophosphates (OPs), which are some of the cholinesterase inhibitor chemicals, are used predominately as pesticides and may induce an oxidative stress status both in rats (Gultekin et al. 2000; Gupta et al. 2001) and human organisms (Banerjee et al. 1999). Moreover, studies have demonstrated the effects of OPs on lipid peroxidation in rat brains (Verma 2001) and human erythrocytes (Gultekin et al. 2000). However, the exact mechanism by which OPs induce oxidative damages is not fully understood (Abdollahi et al. 2004).

Oximes are compounds generally used to reverse the AChE inhibition caused by OPs (Worek 2004). Since studies have proposed a link between organophosphate poisoning and changes in human cellular antioxidant systems (Banerjee et al. 1999), researches on compounds acting as cholinesterase reactivators with antioxidant properties may be a good approach for OP intoxication treatments. Conversely, to the best of our knowledge, there are no data concerning possible antioxidant properties of oximes.

Therefore, the aim of this study was to examine, *in vitro*, the capacity of 3-(phenylhydrazone) butan-2-one oxime (Figure 1) as a potential antioxidant agent using lipid peroxidation (TBARS determination) as endpoint of *in vitro* toxicity both under basal (auto-oxidation of the homogenates) and in the presence of pro-oxidants, including hydrogen peroxide, Fe^{2+}, and malonate (Puntel et al. 2005a, b). The possible antioxidant activity of this oxime was also determined by the analysis of its iron chelating properties (Bucber et al. 1983; Minotti and Aust 1987). A chemical system of desoxyribose degradation (Halliwell and Gutteridge 1981; Gutteridge 1981) and benzoate hydroxylation (Gutteridge 1987) were used. Moreover, we evaluated the *in vitro* $DPPH^{\bullet}$ (Choi et al. 2002) and nitric oxide (NO) (Marcocci et al. 1994) radical scavenger activity of the oxime. *Ex vivo* experiments were

carried out using δ-aminolevulinate dehydratase (δ-ALA-D) [a sulfhydryl enzyme that can be modified by different pro-oxidant situations (Folmer et al. 2003; Perottoni et al. 2005)], thiol levels, and lipid peroxidation as markers of oxime toxicity. These markers were analyzed under basal conditions and in the presence of pro-oxidants.

MATERIAL AND METHODS

Chemicals

Oxidant agents such as malonate, hydrogen peroxide, and iron sulphate ($FeSO_4$) were obtained from local suppliers. σ-phenantroline was obtained from Merck (Darmstadt, Germany). The other reagents used were supplied by Sigma-Aldrich chemical CO (St. Louis, MO).

Synthesis of 3-(phenylhydrazone) butan-2-one oxime

The 3-(phenylhydrazone) butan-2-one oxime was prepared by simple mixture and reflux for 3 hours of 1 mol diacetylmonoxime with 1 mol of phenylhydrazine chloride both dissolved in a mixture of EtOH-H_2O (2:1, v/v) and 0.5 mL of sodium acetate 6 M. On heating, a dark orange product was formed, collected by filtration, washed with water, and dried in vacuum (yield 70%, mp 190°C).

For all experimental procedures the oxime solutions were prepared in dimethyl sulfoxide (DMSO).

In vitro experiments

Assays without tissues in test tubes

Iron chelating properties

In order to examine iron chelating properties of 3-(phenylhydrazone) butan-2-one oxime we used the σ-phenantroline method (Bucber et al. 1983; Minotti and Aust, 1987) according to Puntel et al. (2005a). The method is based on the reaction of free Fe^{2+} with σ-phenantroline forming a colored complex. First, the mixture containing Fe^{2+} (150 μM) and oxime solutions, or its vehicle (DMSO) was allowed to form a complex for 5 min. Afterwards, a solution of σ-phenantroline was added to the mixture (62.5 μg/mL equivalent to 0.25%). The formation of complexes between Fe^{2+} and oxime was estimated by a decrease in the color reaction at 510 nm when compared to a control tube containing Fe^{2+} and σ-phenantroline alone. The values are expressed in percentage of control (without oxime). The solutions were prepared freshly for each experiment.

Deoxyribose degradation assay

The deoxyribose degradation assay was performed according to Puntel et al. (2005a).

Briefly, the reaction medium was prepared containing the following reagents at the final concentrations indicated: oxime (concentrations indicated in the figures), deoxyribose 3 mM, potassium phosphate buffer 0.05 mM, 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 37ºC for 30 min and stopped by the addition of 0.8 mL of trichloroacetic acid (TCA) 2.8% followed by the addition of 0.4 mL of thiobarbituric acid (TBA) 0.6 %. Next, the medium was incubated at 100ºC for 20 min and the absorbance was recorded at 532 nm (Halliwell and Gutteridge 1981; Gutteridge 1981). Standard curves of malondialdehyde (MDA) were made in each experiment. The values are expressed as percentage of control values.

$DPPH^{\bullet}$ radical scavenging activity assay

The measurement of the oxime scavenger activity against the stable radical $DPPH^{\bullet}$ (1,1-diphenyl-2-picrylhydrazyl) was performed in accordance with Choi et al. (2002). Briefly, 85 μM $DPPH^{\bullet}$ was added to a medium containing different oxime concentrations. The mediums were incubated for 30 min at room temperature. The decrease in absorbance measured at 518 nm depicted the scavenger activity of the oxime against $DPPH^{\bullet}$. Ascorbic acid was used as positive control to determine the maximal decrease in $DPPH^{\bullet}$ absorbance. The values are expressed in percentage of inhibition of $DPPH^{\bullet}$ absorbance in relation to the control values without oxime (ascorbic acid maximal inhibition was considered 100% of inhibition).

Benzoate hydroxylation assay

Benzoate hydroxylation was determined by the reaction between hydroxyl radicals and benzoate producing fluorescent dihydroxybenzoate as described by Gutteridge (1987). Hydroxyl radicals were generated by a reaction medium containing hydrogen peroxide and Fe^{3+}. Briefly, an iron-bipyridyl complex was freshly prepared by mixing 2 mM bipyridyl and 2 mM $FeCl_3$ in a ratio 4:1. Benzoate at 2 mM final concentration was added in a medium containing 0.02 mM of phosphate-buffered saline (0.15 M NaCl/0.1 M sodium phosphate buffer, pH 7.4); 0.1 mL of iron-bipyridyl complex (final concentrations of 0.16 mM bipyridil and 0.04 mM $FeCl_3$) and a range of oxime concentrations. The reaction was started by the addition of 0.1 mL of 5 mM H_2O_2. The tubes were incubated for 1 hour at 37°C. After the incubation period, 0.2 mL of TBA 0.6%, followed by 0.2 mL of TCA 2.8%, were added. The tubes were incubated for 10 min at 100 °C in water bath. The tube contents were extracted with n-butanol and after a brief centrifugation (1,800 x g for 10 min) the upper layer was

measured spectrofluorimetrically (Excitation at 305 nm and Emission at 408 nm, and both slit widths used were at 5 nm. The values are expressed in fluorescence units.

Nitric oxide (NO) scavenging assay

Scavenging of NO was assessed by incubating sodium nitroprusside (SNP) (5 mM, in PBS) with different concentrations of the oxime at 25 °C. After 120 min, 0.5 mL of incubation solution was sampled and mixed with 0.5 mL of Griess reagent (Green et al. 1981). The absorbance was measured at 550 nm. The amount of nitrite was calculated from standard curve constructed with sodium nitrite. A curve of sodium nitrite, constructed in presence of oxime in order to verify its interaction with nitrite, depicted no interference of oxime in 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 oxime as an index of its NO scavenger activity (Marcocci et al. 1994).

Assays with tissues

Tissue preparation

Brain tissue was obtained from untreated adult *swiss albino* mice from our own breeding colony. The animals were maintained in an air-conditioned room (20- 25ºC) under a 12 hour light/dark cycle, with lights on at 7:00 am, and with water and food ad libitum. All the experimental procedures performed were conducted according to the guidelines of the Committee of Ethics in Research of the Federal University of Santa Maria, Brazil. After anesthesia, the mice were euthanized by decapitation and the brain was quickly removed, placed on ice, and homogenized within 10 min in 10 volumes of cold saline 150 mM. The homogenate was centrifuged at 4,000 x g at 4°C for 10 min to yield a low speed supernatant fraction (S1) that was used for thiobarbituric acid reactive substance measurement (TBARS) and δ-ALA-D activity assays. Lipid of eggs was prepared from chicken eggs. The egg yolks were weighed and mixed with a solvent mixture [iso-propanol and hexane (3:2); 10 mL of solvent/g of yolk]. The mixture was evaporated at a maximum temperature of 60°C. Total lipid of egg yolk extract was determined by a commercial kit (Bioclin obtained from local suppliers) with few modifications and it was used for TBARS assay.

Lipid peroxidation assay

Lipid peroxidation was determined by measuring TBARS as described by Ohkawa et al. (1979) in lipid extracts from egg yolk and in S1 brain homogenates. Aliquots of 100 μL of lipids or 200 μL of S1 were mixed to incubating medium containing Tris-HCl (0.01 mM), oxime at indicated concentrations (see figure legends), and pro-oxidant agents when

specified, and then incubated at 37ºC for 60 min. The reaction was stopped by adding 0.5 mL of acetic acid buffer, and lipid peroxidation products were measured by the addition of 0.5 mL of TBA 0.6%. Tubes were then incubated in boiling water for 60 min and their contents were extracted with n-butanol before spectrophotometric analysis. TBARS levels were measured at 532 nm using a standard curve of MDA. The pro-oxidants malonate, iron, and hydrogen peroxide were added as positive control for lipid peroxidation.

δ-ALA-D activity assay

The brain enzyme was assayed according to Sassa method (1982) by measuring the rate of product porphobilinogen (PBG) formation. After 10 min of pre-incubation of the enzyme with oxime at 37ºC, in a medium containing 100 mM potassium phosphate buffer, pH 6.8, the enzymatic reaction was initiated by adding the substrate aminolevulinic acid (ALA) to a final concentration of 2.5 mM. The incubation was carried out for 3 hours, at 37ºC, and was stopped by adding 10% TCA containing 10 mM $HgCl_2$. The reaction product was determined using a modified Ehrlich's reagent at 555 nm, with a molar absorption coefficient of 6.1×10^4 for the Ehrlich porphobilinogen salt. Simultaneously, a set of tubes was assayed using a similar incubation medium, except that 2 mM DTT was also added to observe the possible reactivation of the δ-ALA-D activity. The enzyme activity was expressed in nmol of PBG /hour/mg of protein.

***Ex vivo* experiments**

Animal Treatment

Adult *swiss albino* mice from our own breeding colony were maintained in an air-conditioned room (20- 25ºC) under a 12 hour light/dark cycle, with lights on at 7:00 am, and with water and food ad libitum. All the experimental procedures performed were conducted according to the guidelines of the Committee of Ethics in Research of the Federal University of Santa Maria, Brazil. The animals received a single subcutaneous injection of the oxime dissolved in DMSO in different doses (0.5, 1, 5, 10, 100, 200, 300, 400 or 500 mg/kg). Control animals received DMSO at 10 mL/kg. To determine the potential lethality of 3-(phenylhydrazone) butan-2-one oxime, animals were observed for up to 72 h after compound administration. LD_{50} was calculated using "GraphPad Software" (GraphPad software, San Diego, CA). After this period, animals were euthanized by cervical dislocation. The brain of all experimental groups was quickly removed, placed on ice, and homogenized within 10 min, in 10 volumes of cold saline 150 mM. The homogenate was centrifuged at 4,000 x g at 4°C for 10 min to yield a low speed supernatant fraction (S1) that was used for *ex vivo* assay

for non-protein thiol, TBARS and δ-ALA-D activity measurements.

Non protein thiol measurement (NPSH)

To determine NPSH, 500 μL of 10% TCA was added to 500 μL of S1 brain homogenates. After centrifugation (4,000 × *g* at 4 ◦C for 10 min), the protein pellet was discarded and free –SH were determined in the clear supernatant (which was previously neutralized with 0.1 M NaOH) according to Ellman (1952).

TBARS and δ-ALA-D activity measurements

The assays were conduced as described above to the *in vitro* experiments.

Protein determination

The protein content was determined according to Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

Statistical analysis

Data were analyzed by one-way ANOVA, followed by Tukey multiple range test when appropriate. Differences between groups were considered significant when $p \leq 0.05$.

RESULTS

***In vitro* results**

The induced Fenton reaction (Fe^{2+} + H_2O_2) and Fe^{2+} or H_2O_2 oxidant species, when studied in separate, were able to stimulate deoxyribose degradation. However the oxime did not modify this parameter (Figure 2). Besides, the benzoate hydroxylation induced by H_2O_2 was not modified by the oxime (Figure 3). 3-(phenylhydrazone) butan-2-one oxime did not present iron (Fe^{2+}) chelating activity (data not show).

Data regarding $DPPH^{\bullet}$ assay suggest that oxime showed a significant $DPPH^{\bullet}$ radical scavenging activity at 25 μM (Figure 4). Moreover, oxime showed a significant NO scavenging activity at 0.5 μM (Figure 5).

Lipid peroxidation, induced by H_2O_2 in brain S1 homogenates, was significantly reduced by oxime at 0.1 μM ($p<0.05$). Besides, the production of TBARS, induced by malonate and iron, was significantly decreased at 0.4 μM of oxime ($p<0.05$) (Figure 6A). However, oxime was not able to decrease the induced TBARS production in lipids from egg yolk (Figure 6B). Furthermore, the 3-(phenylhydrazone) butan-2-one oxime did not modify the δ-ALA-D activity *in vitro* (Figure 7).

***Ex vivo* results**

Animals treated with oxime presented no changes in brain non-protein thiol levels

(Figure 8). Furthermore, oxime exposure was unable to decrease the basal or prevent pro-oxidant induced lipid peroxidation in brain from animals exposed to oxime 72 hours before (Figure 9). Mice treated with oxime depicted no changes in the brain δ-ALA-D activity in all groups tested both in presence or absence of DTT (2 mM) (Figure 10).

The LD_{50} for 3-(phenylhydrazone) butan-2-one oxime was higher than 500 mg/kg (n=4) (data not shown). Furthermore, no groups tested with oxime presented loss of body weight (data not show).

DISCUSSION

The organophosphate (OP) intoxication can be accompanied by an increase in RS generation in biological tissues (Banerjee et al. 1999). Under such conditions, oximes are commonly used as pharmacological agents mainly to reverse the AChE inhibition caused by OPs (Worek 2004). In this context, an oxime able to act against both AChE inhibition and RS generation can be a good approach in treatment protocols.

The results obtained indicate that 3-(phenylhydrazone) butan-2-one oxime possesses a significant *in vitro* antioxidant activity against the oxidative damage induced by different oxidant agents, such as malonate, iron, and hydrogen peroxide, at nano molar concentrations. In brain homogenates, the lipid peroxidation, induced by 5 μM iron, was reduced by 0.1 μM of oxime, a concentration 50 fold lower than the oxidant agent used here. Moreover, the damage induced by 500 μM hydrogen peroxide and 4 mM malonate was reduced with 0.4 μM of oxime, i.e., concentrations of 1,250 and 10,000 fold lower than the oxidant agents used here, respectively.

Taking into account the results observed in the TBARS assay in the presence of S1 homogenates, we believe that the effects of lipid peroxidation neutralization could involve the oxime and/or an additional effect of some metabolite produced. This seems evident since the oxime was unable to prevent the induced TBARS production in a medium with lipids alone. Moreover, we observed a significant decrease in basal lipid peroxidation at 0.1 μM, which corroborates the hypothesis of some metabolic process on antioxidant action mechanism of the oxime. Furthermore, the deoxirybose degradation caused by oxidant agents such as iron (5 μM), hydrogen peroxide (500 μM), and iron plus hydrogen peroxide was not affected by oxime. The fact that we did not test concentrations higher than 1 μM of the oxime in deoxyribose degradation assay (Figure 2) and also on lipid peroxidation in lipid of egg yolk assay (Figure 6B) was due to our intent in explain the strong antioxidant activity showed by the oxime in experiments with mice brain homogenates.

In order to identify a possible mechanism for the effect of oxime in the induced lipid peroxidation in brain homogenates, we searched for its scavenging capacity in different protocols. Some data have pointed to an important mechanism which would accelerate the lipid peroxidation catalyzed by metal ions, such as iron, via the Fenton reaction. This could give alkoxyl and peroxyl radicals, both of which can lead to hydroxyl radical formation (Halliwell and Gutteridge 1984). Antioxidants or chelating agents effectively block iron-mediated oxidant damage (Graf et al. 1987). Some oximes have attracted considerable interest due to their possible capacity to form metal complexes (Adam, 1997). However, we did not identify a significant iron chelating activity of the oxime.

Furthermore, we analyzed the hydroxyl scavenging activity of the oxime using a $DPPH^{\bullet}$ assay. A stable radical, 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$), has been used as a convenient method for the antioxidant assay of biological materials, such as cysteine, glutathione, ascorbic acid, tocopherol, and polyhydroxy aromatic compounds (Nishizawa et al. 2005). $DPPH^{\bullet}$ has been investigated as a reactive hydrogen acceptor (Braude et al. 1954), and the mechanism of $DPPH^{\bullet}$-scavenging activity involves reductive hydrogen transference between donors and $DPPH^{\bullet}$. The data obtained suggest that the oxime depicted a significant $DPPH^{\bullet}$ radical scavenging activity at 25 μM (Figure 4). The reason for the higher concentration used in $DPPH^{\bullet}$ radical scavenging assay was that no effects were observed at the concentrations which presented significant effects in abolish the *in vitro* basal and pro-oxidant induced lipid peroxidation in brain homogenized. Thus the antioxidant effects of the oxime might not be correlated integrally to its $DPPH^{\bullet}$ radical scavenging activity.

Conversely, oxime was unable to prevent the hydroxyl radical formation through the Fenton reaction elicited in the benzoate hydroxylation assay. Thus, the absence of effects in dihydroxylbenzoate formation along with the data obtained from both the deoxyribose degradation assay and the TBARS induced in lipids clearly indicates that the oxime did not interfere in the Fenton reaction. Given that the free radical nitric oxide could participate in oxidative damage as an effective molecule (Ignaro 1990), we searched for a possible oxime nitric oxide scavenging activity. Our findings pointed to a significant capacity of oxime to neutralize the nitric oxide and thus the possible formation of reactive nitrogen species. Although a significant NO scavenging activity was reached at nano molar concentration (0.5 μM), we believe that a metabolic process could be involved in the improvement of its antioxidant capacity.

Some important cellular enzymatic systems such as δ-ALA-D can be inhibited by thiol oxidizing radicals (Folmer et al. 2003; Farina et al. 2001; Perottoni et al. 2005). δ-ALA-D activity inhibition could be a good marker for oxidative stress (Maciel et al. 2000). The absence of significant effects on δ-ALA-D activity indicates that oxime does not affect the essential –SH groups on the active site of the enzyme and does not increase oxidative stress either.

An unexpected result comparing the *in vitro* and *ex vivo* experiments showed that oxime was unable to prevent the induced lipid peroxidation in the *ex vivo* experiments. The reason for this contradictory result may be the plasmatic life-time of 3-(phenylhydrazone) butan-2-one. Previous studies reported a short plasmatic life-time of pralidoxime, a common clinically used oxime, of about 75 min (Howland and Aaron 1999). Other studies have reported that the protective capacity of pralidoxime to reactivate the dimethoate-inhibited erythrocyte AChE was reduced by up to 50% in 6 hr and disappeared almost completely in 24 hr (Rios et al. 2005). The *in vivo* experiments indicate that 3-(phenylhydrazone) butan-2-one oxime presents a small toxicity (s.c.) (LD_{50} higher than 500 mg/kg) in mice when compared with other oximes, like obidoxime (i.m. LD_{50}=188.4 mg/kg) (Ševelová et al. 2004) and pralidoxime (i.m. LD_{50}=180 mg/kg or i.p. LD_{50}=155 mg/kg) (Arena 1979). Furthermore, the animals presented no loss of body weight during the experiment (data not shown). The activity of δ-ALA-D did not change in oxime treated animals either. This datum emphasizes the fact that 3-(phenylhydrazone) butan-2-one oxime does not affect the –SH groups located at the active center of δ-ALA-D, similar to the *in vitro* results. Furthermore, the pre-treatment of animals with oxime is unable to alter the –SH buffer in animal brains. The preservation of NPSH levels could indicate that oxime does not act *in vivo* on the biological –SH groups.

In conclusion, our data show that, although the results obtained *in vitro* in assays without tissues in test tubes point to a significant oxime scavenger activity of the $DPPH^{\bullet}$ and NO radicals, a metabolic process could be involved in the improvement of its antioxidant capacity. This seems evident based in the results depicted *in vitro* in assays with tissues, since oxime was unable to prevent the induced TBARS production in a medium composed essentially by lipids of eggs or deoxyribose, while elicited a strong antioxidant capacity in induced TBARS in S1 brain homogenates of untreated mice (0.1 μM for hydrogen peroxide and 0.4 μM for malonate and iron). Furthermore, *ex vivo* results also indicate that oxime and its metabolites are probably not toxic at all. Although our *ex vivo* protocol did not allow us to detect a protection against oxidant agents, this lack of protection may have been caused by the

short half life of the compound. We believe that other experimental procedures could demonstrate that oxime is an effective protector against RS damage *in vivo*.

AKNOWLEDGMENTS

Work Supported by the FINEP research grant "Rede Instituto Brasileiro de Neurociência (IBN-Net)" # 01.06.0842-00. P.G. receives a fellowship by CNPq/PIBC/UFSM. Additional support giving by CNPq, FAPERGS and CAPES.

REFERENCES

Abdollahi M, Ranjbar A, Shadnia S, Nikfar S, Rezaie A, (2004) Pesticides and oxidative stress: a review. Med Sci Monit 10(6):141-147

Adam J P, (1997) Imines, enamines and oximes. Contemp Org Synth 4(6):517-543

Arena J M, (1979) Poisoning, Toxicology, Symptoms, Treatments, 4th edn. Springfield Ill, Illinois, USA, Charles C.Thomas, p. 133

Banerjee B D, Seth V, Bhattacharya A, Pasha S T, Chakraborty A K, (1999) Biochemical effects of some pesticides on lipid peroxidation and free-radical scavengers. Toxicol Lett 107:33–47

Braude B A, Brook A G, Linstead R P, (1954) Hydrogen transfer. Part V. Dehydrogenation reactions with diphenylpicrylhydrazyl. J Chem Soc 3574-3578

Bucber J R, Tien M, Morehouse L A, and Aust S D, (1983) Redox cycling and lipid peroxidation*:* The central role of iron chelates. Fundam Appl Toxicol 3:222-226

Choi C W, Kim S C, Hwang S S, Choi B K, Ahn H J, Lee M Y, Park S H, Kim S K, (2002) Antioxidant activity and free radical scavenging capacity between Korean medicinal plants and flavonoids by assay-guided comparison. Plant Sci153:1161-1168

Ellman G L, (1952) Tissue sulfhydryl groups. Arch Biochem Biophys 82:70–7

Farina M, Folmer V, Bolzan R C, Andrade L H, Zeni G, Braga A L, Rocha J B T, (2001) Selenoxides inhibit δ-aminolevulinic acid dehydratase. Toxicol Lett 119:27–37

Folmer V, Soares J C M, Gabriel D, Rocha J B T, (2003) A high fat diet inhibits δ-aminolevulinate dehydratase and increases lipid peroxidation in mice (*Mus musculus*). J Nutr 133(7):2165-2170

Graf E, Empson K L, Eaton J W, (1987) Phytic acid: a natural antioxidant. J Biol Chem 262:11647–11650

Green L C, Wanger D A, Glogowski J, Skipper P L, Wishnok J S, Tannenbaum S R, (1981) Analysis of nitrate, nitrite, [^{15}N] nitrate in biological fluids. Anal Biochem 126:131–138

Gultekin F, Ozturk M, Akdogan M, (2000) The effect of organophosphate insecticide chlorpyrifos-ethyl on lipid peroxidation and antioxidant enzymes (*in vitro*). Arch Toxicol 74:533-568

Gutteridge J M C, (1987) Ferrous-salt-promoted damage to deoxyribose and benzoate. The increased effectiveness of hydroxyl-radical scavengers in the presence of EDTA. Biochem J 243(3):709–714

Gutteridge J M C, 1981. Thiobarbituric acid-reactivity following iron-dependent free-radical damage to amino acids and carbohydrates. FEBS Lett 128:343-346

Gutteridge J M C, Halliwell B, (1994) Antioxidants in nutrition, Health and Disease. New York: Oxford University Press. 111–123

Halliwell B, (2006) Oxidative stress and neurodegeneration: Were are we now? J Neurochem 97:1634–1658

Halliwell B and Gutteridge J M C, (1981) Formation of a 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 C M, (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J 219:1–14

Halliwell B, Zhao K and Whiteman M, (1999) Nitric oxide and peroxynitrite. The ugly, the uglier and the not so good: a personal view of recent controversies. Free Radic Res 31:651–669

Howland M A, Aaron C, (1999) Pralidoxime. In: Goldfrank, L., Flomenbaum, N., Weisman, R., Howland, M.A., Hofman, R. (Eds.), Emergencies Toxicology. Applenton & Lange, USA. 1445-1449

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

Maciel E M, Bolzan R C, Braga A L, Rocha J B T, (2000) Diphenyl diselenide and diphenyl ditelluride differentially affects d-aminolevulinate dehydratase from liver, kidney and brain of mice. J Biochem Mol Toxicol 14:310-319

Marcocci L, Maguire J J, Droy-Lefaix M T, Packer L, (1994) The nitric oxide-scavenging properties of Ginkgo biloba extract EGb 761. Biochem Biophys ResCommun 201:748–755

Minotti G, and Aust S D, (1987) An investigation into the mechanism of citrate-Fe^{2+}-dependent lipid peroxidation. Free Radic Biol Med 3, 379- 387.

Nishizawa M, Kohno M, Nishimura M, Kitagawa A, Kiwano Y, (2005) Non-reductive scavenging of 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) by peroxyradical: a useful method for quantitative analysis of peroxylradical. Chem Pharm Bull (Tokyo) 53(6):714-716

Nogueira C W, Zeni G, Rocha J B T, (2004) Organoselenium and Organotellurium Compounds: Toxicology and Pharmacology. Chem Rev 104:6255-6285

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

Perottoni J, Meotti F C, Folmer V, Pivetta L, Nogueira C W, Zeni G, Rocha J B T, (2005) Ebselen and diphenyl diselenide do not change the inhibitory effect of lead acetate on delta-aminolevulinate dehidratase. Environ Toxicol Pharmacol 19:239–248

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

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

Puntel R L, Roos D H, Paixão M W, Braga A L, Zeni G, Nogueira C W, Rocha J B T, (2007) Oxalate modulates thiobarbituric acid reactive species (Tbars) production in supernatants of homogenates from rat brain, liver and kidney: Effect of diphenyl diselenide and diphenyl ditelluride. Chem Biol Interact 165:87-98

Rios J C, Repetto G, Galleguillos I, Jos A, Peso A D, Repetto M, (2005) High concentrations of pralidoxime are needed for the adequate reactivation of human erythrocyte acetylcholinesterase inhibited by dimethoate *in vitro*. Toxicol *In Vitro* 19(7):893-897

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

Ševelová L, Kuča K, Krejčová-Kunešová G, (2004) Antidotal treatment of GF-agent intoxication in mice with bispyridinium oximes. Toxicol 207:1-6

Urso M, Clarkson P M, (2003) Oxidative stress, exercise, and antioxidant supplementation. Toxicol 189:41-54

Verma R S, (2001) Chlorpyrifos-induced alterations in levels of thiobarbituric acid reactive substances and glutathione in rat brain. Indian Exper Biol 39:174–77

Worek F, Thiermann H, Szinicz L, Eyer P, (2004) Kinetic analysis of interactions between human acetylcholineserase, structurally different organophosphorus compounds and oximes. Biochem Pharmacol 68:2237–2248

Figure 1:

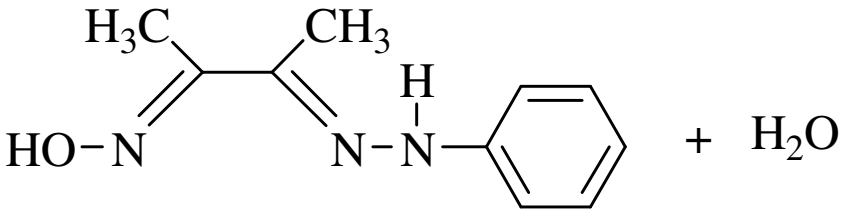

Figure 1: **Chemical structure of 3-(phenylhydrazone) butan-2-one oxime**.

Figure 2:

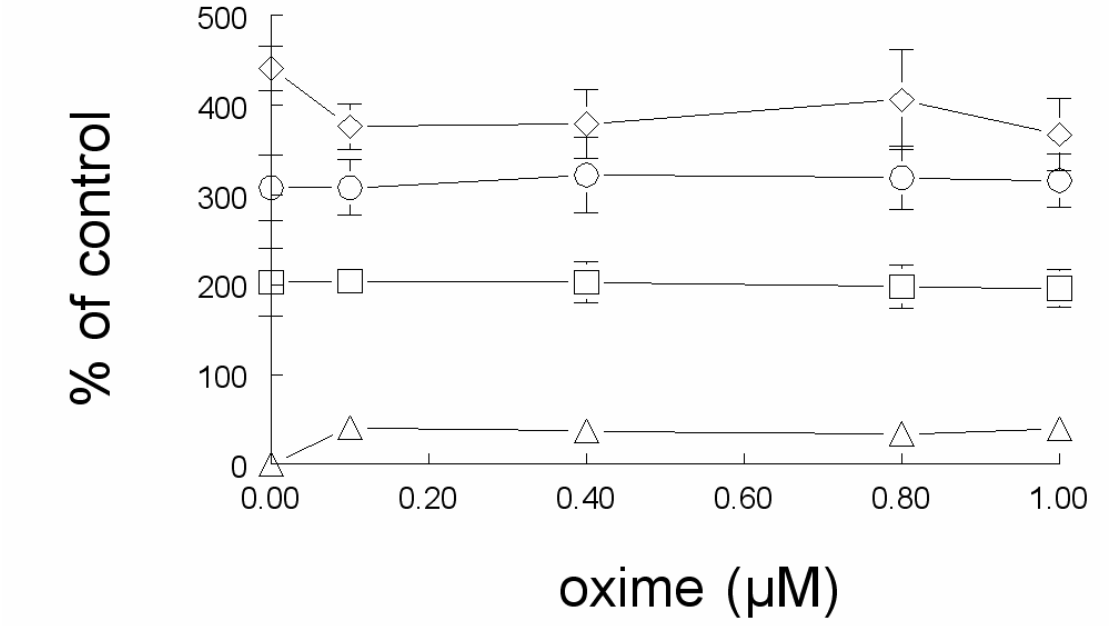

Figure 2: **Effects of oxime on deoxyribose degradation.** The (-Δ-) indicates basal conditions, (-○-) hydrogen peroxide at 500 µM, (-□-) iron at 5 µM and (-◊-) Fe^{2+} 5 µM plus hydrogen peroxide 500 µM conditions. The values are expressed as percentage of control values. The mean control value is 0.548±0.003 µM MDA/g of deoxyribose. Data are presented as mean ± SEM (n=3).

Figure 3:

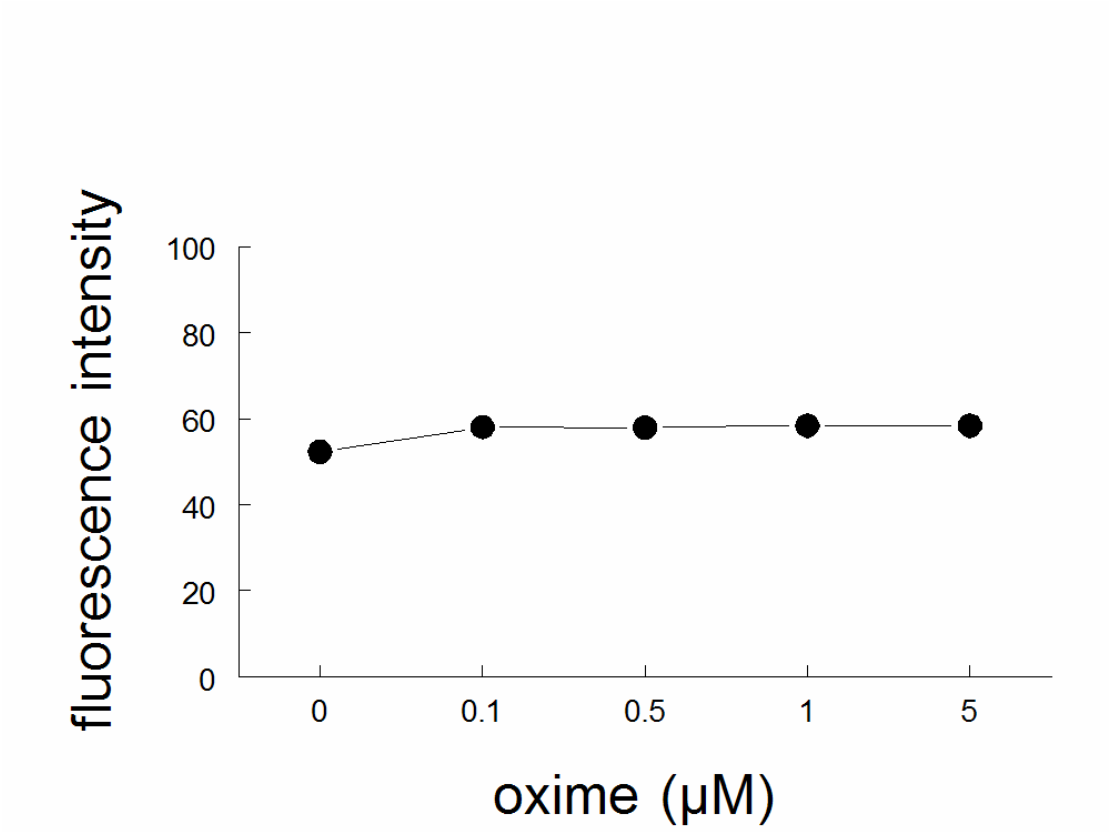

Figure 3: **Effects of oxime on benzoate hydroxylation**. Effects of 3-(phenylhydrazone) butan-2-one oxime on benzoate hydroxylation by measuring the dihydroxybenzoate products formation. The values are expressed in fluorescence units. Data are presented as mean ± SEM (n=3).

Figure 4:

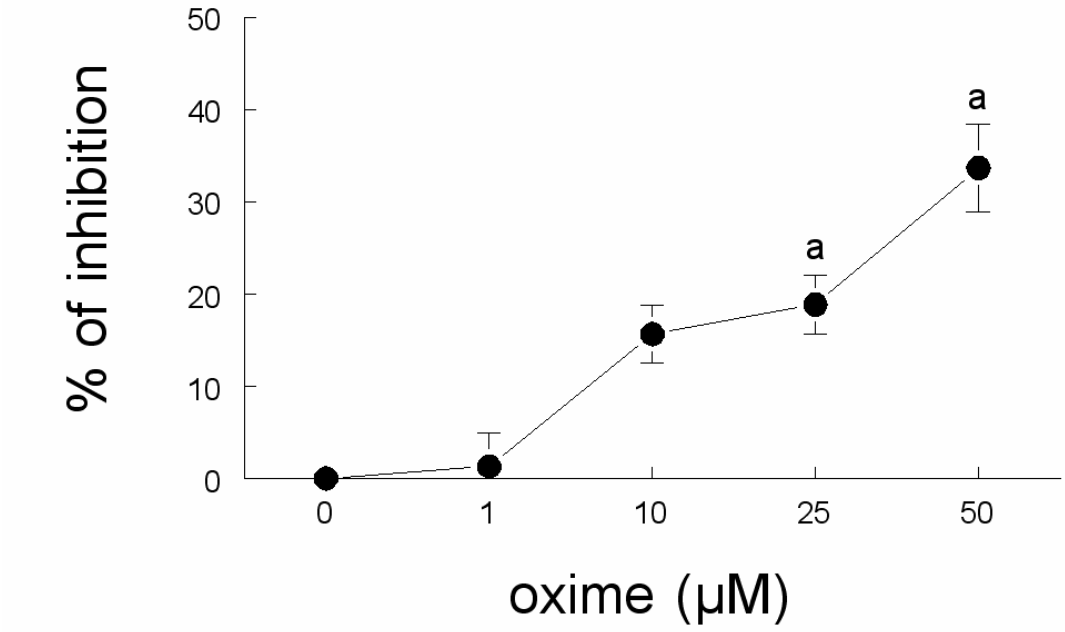

Figure 4: **DPPH• radical scavenging activity of oxime**. Effect of 3-(phenylhydrazone) butan-2-one oxime on DPPH radical scavenging activity. The values are expressed in percentage of inhibition in relation to control without oxime or ascorbic acid. The mean control value is 0.658±0.02 ABS. Data are presented as mean ± SEM (n=3). ([a]) Indicates $p<0.05$ from respective control by Tukey´s multiple range test.

Figure 5:

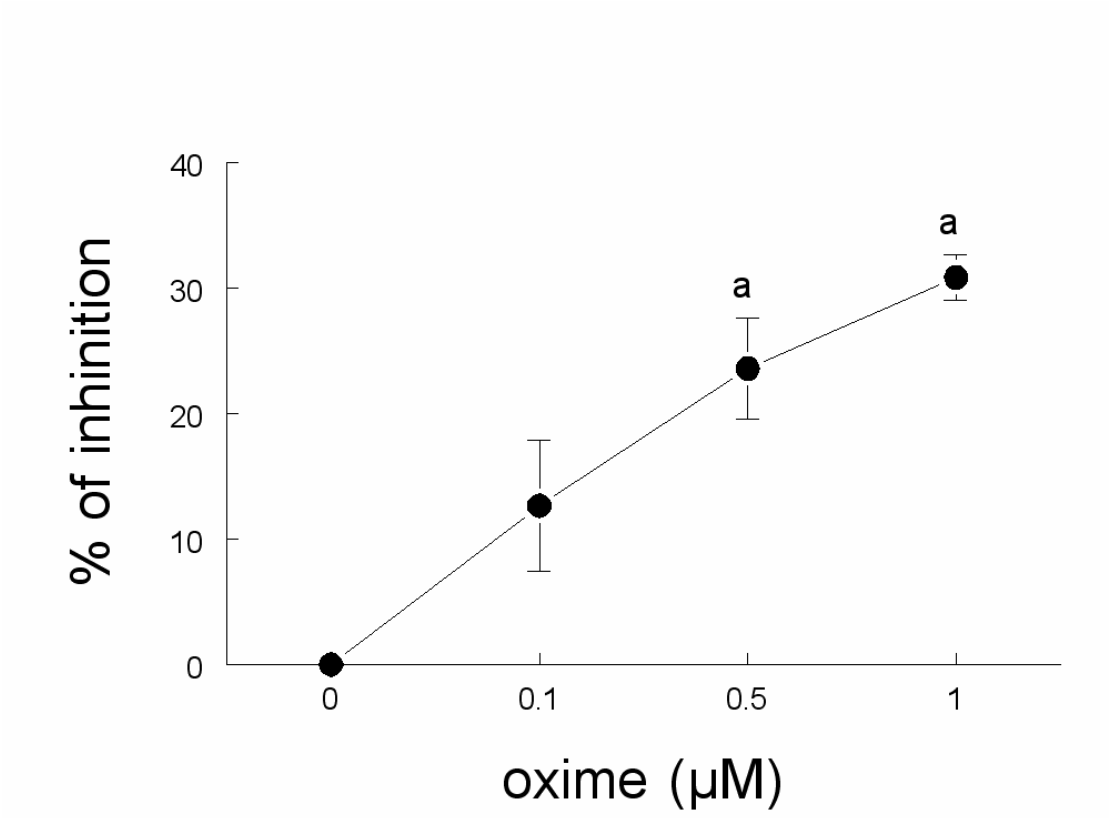

Figure 5: **Effects of oxime on nitric oxide (NO) scavenging assay**. Effects of 3-(phenylhydrazone) butan-2-one oxime on NO scavenging activity. The values are expressed in percentage of inhibition in relation to control without oxime. The mean control value is 18.399±1.628 µM of nitrite. Data are presented as mean ± SEM (n=3). ([a]) Indicates $p<0.05$ from respective control by Tukey´s multiple range test.

Figure 6A:

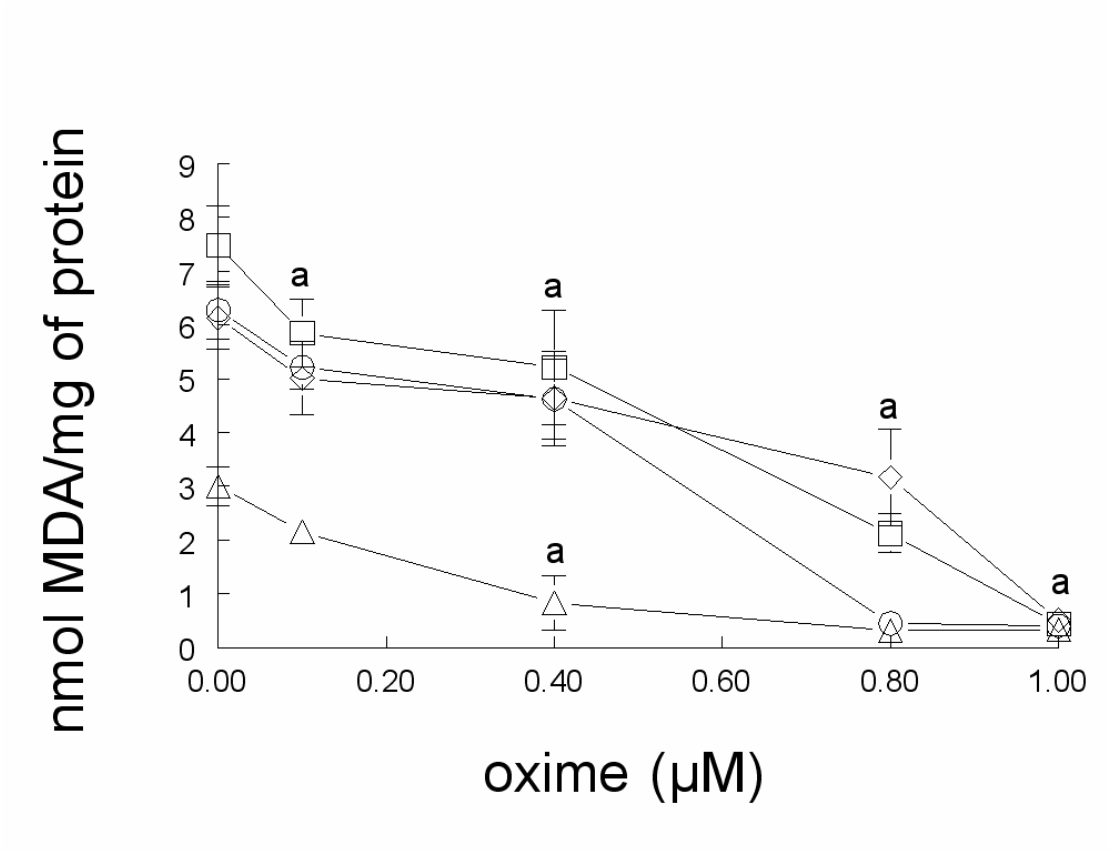
nmol MDA/mg of protein
9
8
7
6
5
4
3
2
1
0
a
a
a
a
a
0.00
0.20
0.40
0.60
0.80
1.00
oxime (μM)

Figure 6B:

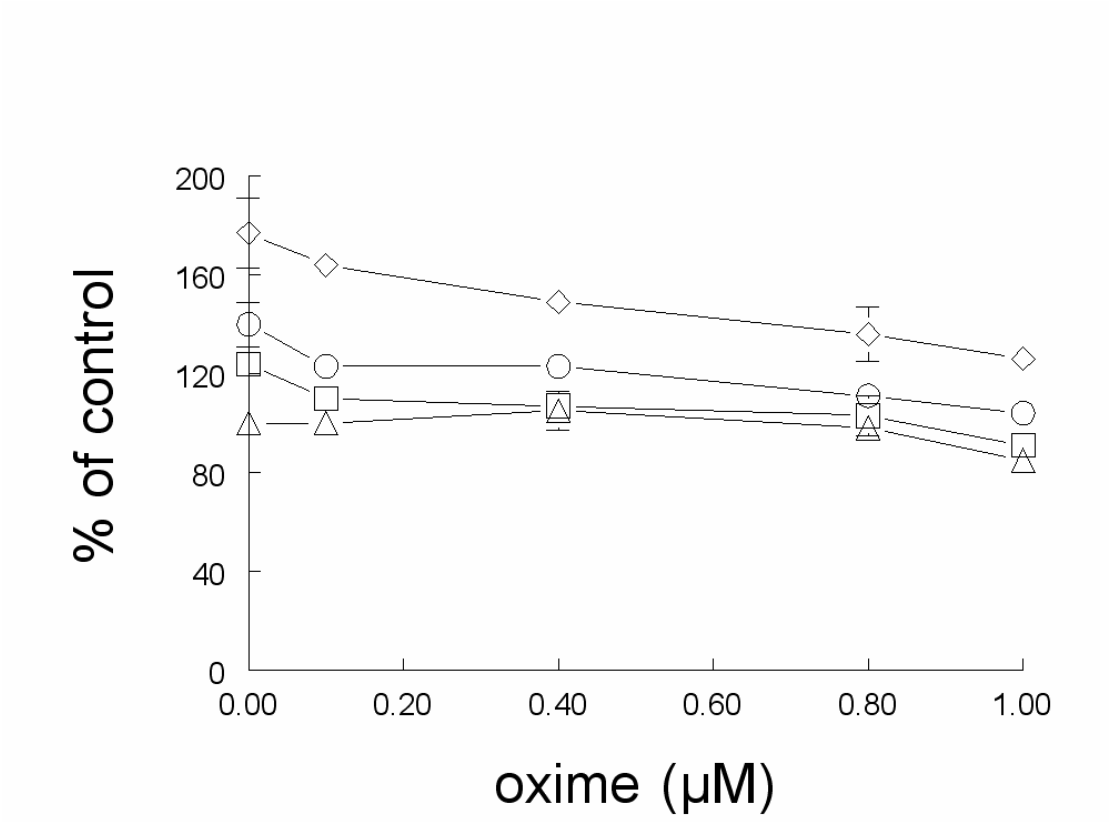

Figure 6: **Effects of oxime on basal or oxidant agents induced TBARS production.** Effects of 3-(phenylhydrazone) butan-2-one oxime on basal or oxidant agents induced TBARS production in low-speed supernatant (S1) from brain homogenates (6A), or in lipids from eggs (6B). In figure 6A (-Δ-) indicates control conditions, (-○-) malonate at 4mM, (-□-) iron at 5µM, and (-◊-) hydrogen peroxide at 500µM. In figure 6B (-Δ-) indicates control conditions, (-○-) hydrogen peroxide at 500µM, (-□-),iron at 5µM and (-◊-) iron plus hydrogen peroxide. TBARS levels are expressed as nmol of MDA/mg of protein in 6A and in percentage of control in 6B. The mean control value is 1.66±0.52 µM MDA/g of lipid. Data are presented as mean ± SEM (n=3). ([a]) Indicates $p<0.05$ from respective control by Tukey´s multiple range test.

Figure 7:

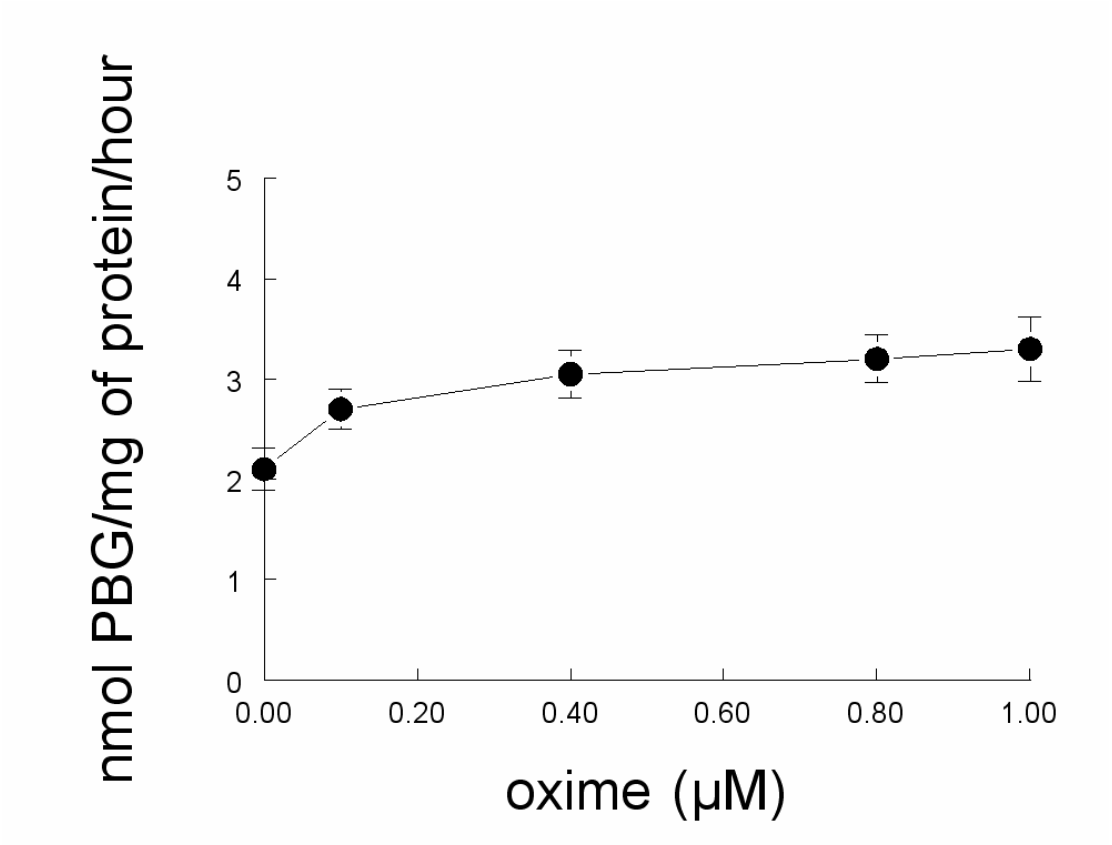

Figure 7: **Effects of oxime on δ-ALA-D activity**. Effects of 3-(phenylhydrazone) butan-2-one oxime on δ-ALA-D activity in low-speed supernatant (S1) from brain homogenates. The specific activity is expressed as nmol PBG formed per hour per mg of protein. Data are presented as mean ± SEM (n=3).

Figure 8:

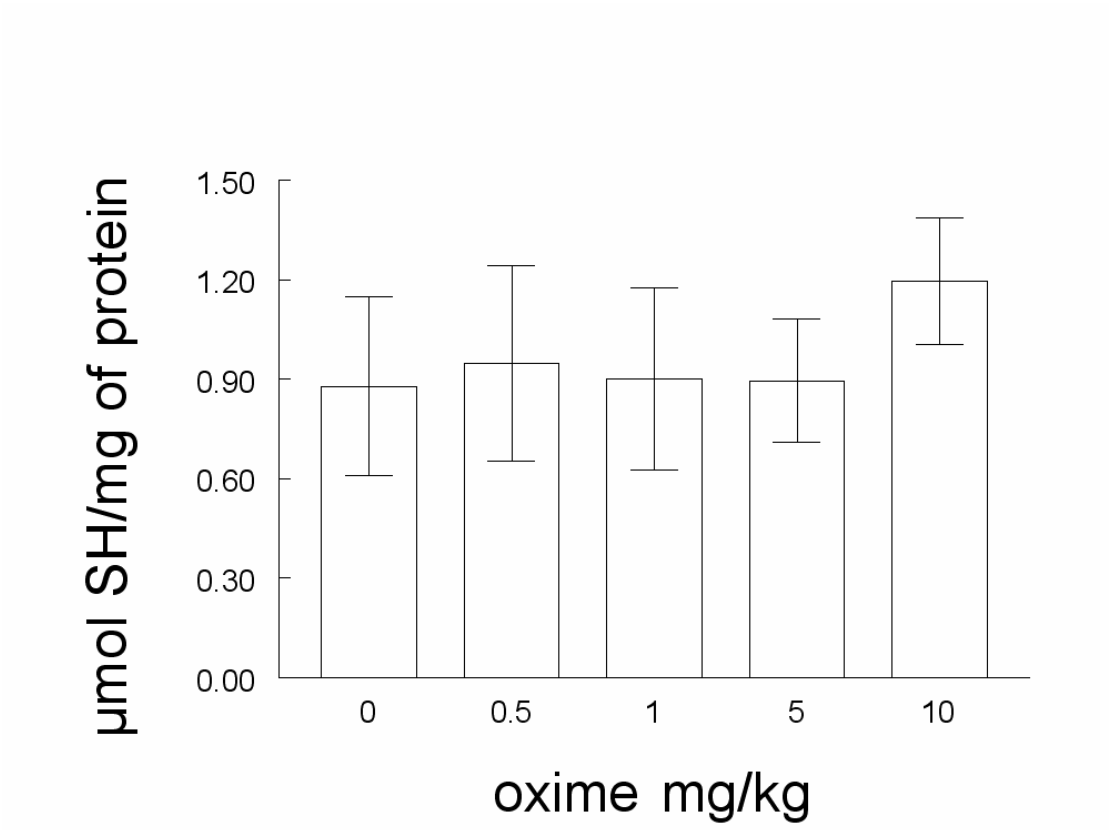

Figure 8: **Effect of oxime treatment on brain non-protein thiol levels**. Effect of the treatment with 3-(phenylhydrazone) butan-2-one oxime in low-speed supernatant (S1) brain non-protein thiol levels. The values are expressed as µmol SH per mg of protein and presented as mean ± SEM (n=4).

Figure 9:

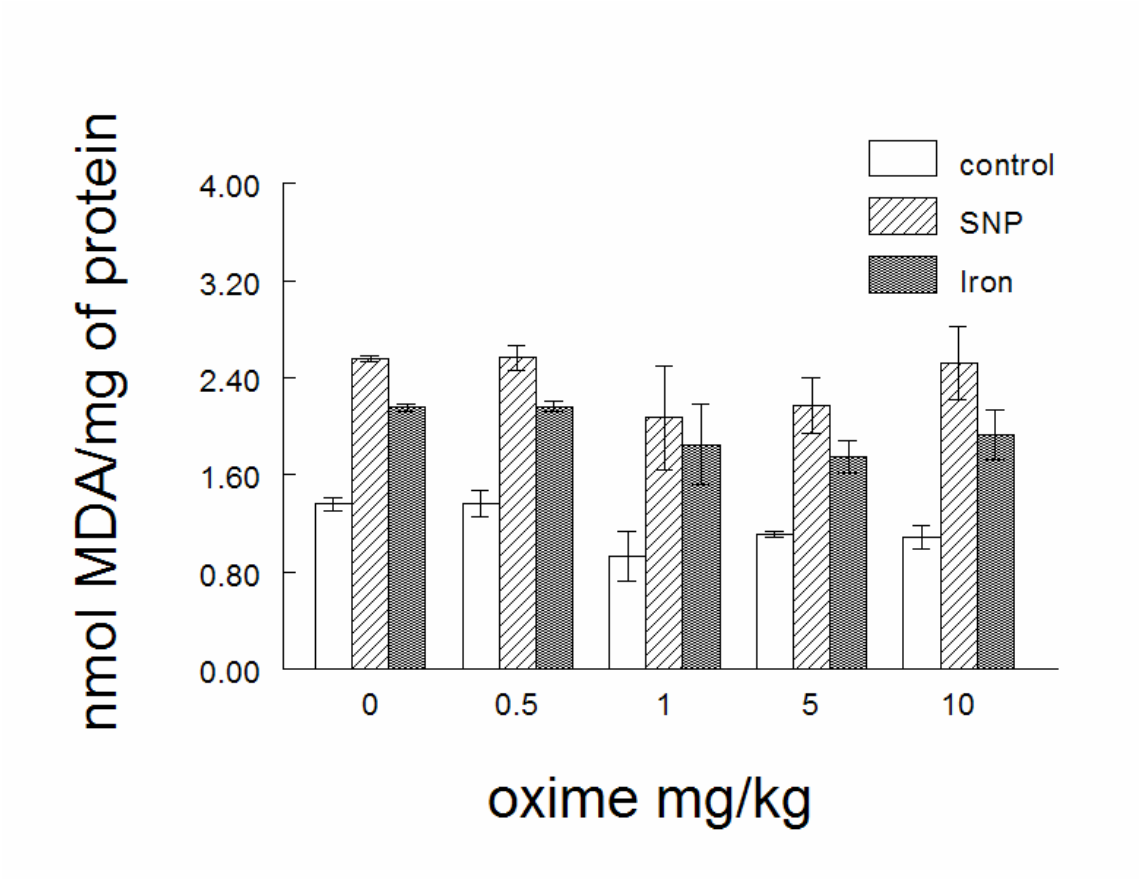

Figure 9: **Effect of oxime treatment on TBARS production levels.** Effect of the treatment with 3-(phenylhydrazone) butan-2-one oxime in low-speed supernatant (S1) brain TBARS production. Sodium nitroprusside (5 µM) and iron (5 µM) were used as pro-oxidants to induce TBARS production. TBARS are expressed as nmol of MDA per mg of protein and presented as mean ± SEM (n=4).

Figure 10:

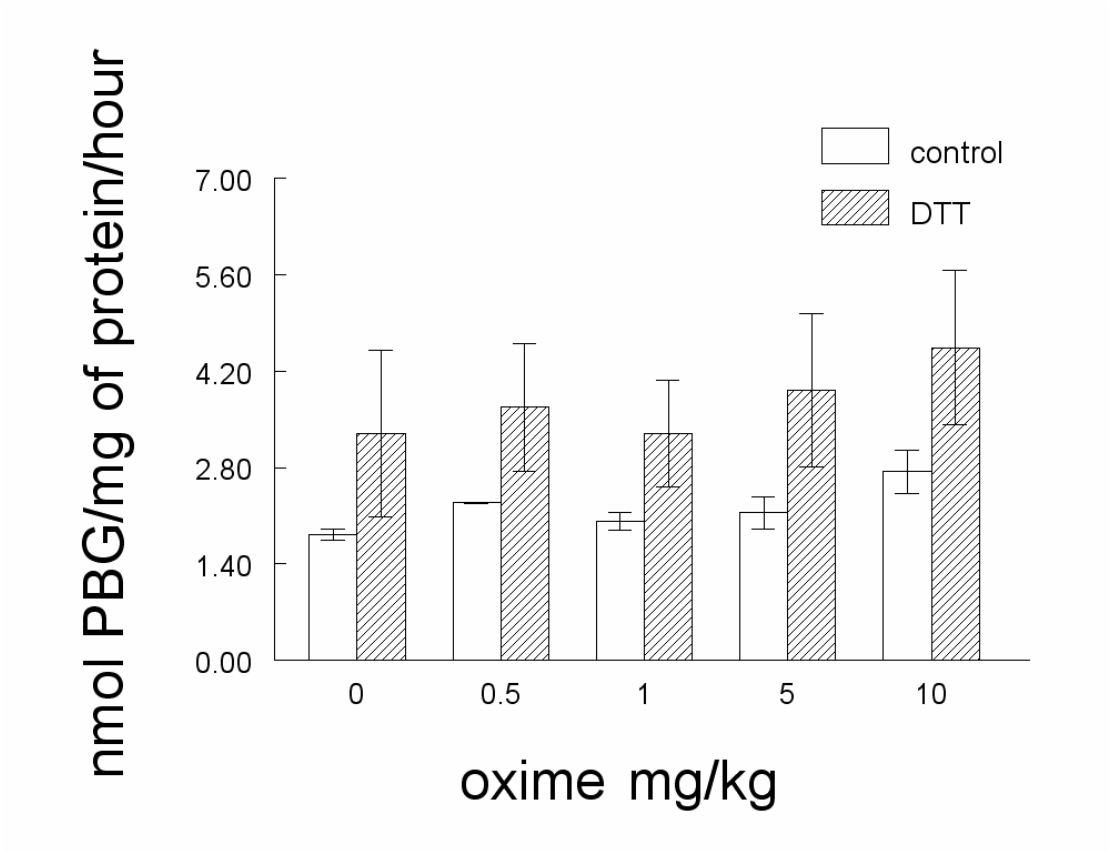

Figure 10: **Effects of oxime treatment in brain δ-ALA-D activity**. Effect of the treatment with 3-(phenylhydrazone) butan-2-one oxime in brain δ-ALA-D activity. The enzyme activity was analyzed in the presence and absence of DTT (2 mM). The values are expressed as nmol PBG formed per hour per mg of protein and presented as mean ± SEM (n=4).

3.2. – BUTANO-2,3-DIONATIOSEMICARBAZONA: UMA OXIMA COM PROPRIEDADES ANTIOXIDANTES

Manuscrito 2

BUTANE-2,3-DIONETHIOSEMICARBAZONE: AN OXIME WITH ANTIOXIDANT PROPERTIES

Gustavo Orione Puntel, Nélson Rodrigues de Carvalho, Priscila Gubert, Aline Schwertner Palma, Maria Ester Pereira, Vanessa Santana Carratu, Leandro Bresolin, João Batista Teixeira da Rocha, Félix A. Antunes Soares

Butane-2,3-dionethiosemicarbazone: an oxime with antioxidant properties

Gustavo Orione Puntel[1], Nelson Rodrigues de Carvalho[1], Priscila Gubert[1], Aline Schwertner Palma[1], Maria Ester Pereira[1], Vanessa Santana Carratu[2], Leandro Bresolin[3], João Batista Teixeira da Rocha[1], Félix A. Antunes Soares[1*]

[1]Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Campus UFSM, Santa Maria, RS, Brasil.

[2]Departamento de Química, Fundação Universidade de Rio Grande, Campus Carreiros, Rio Grande, RS, Brasil.

[3]Universidade Federal do Acre, Campus Cruzeiro do Sul, Cruzeiro do Sul, AC, Brasil.

*CORRESPONDING AUTHOR:

Félix Antunes Soares

Departamento de Química - CCNE - Universidade Federal de Santa Maria

97105-900 - Santa Maria - RS - Brazil

Phone: +55-55-3220-9522

Fax: +55-55-3220-8978

E-mail: felix_antunes_soares@yahoo.com.br

ABSTRACT

Oximes are compounds generally used to reverse the acetylcholinesterase (AChE) inhibition caused by organophosphates (OPs). Besides, there are few data concerning possible antioxidant properties of oximes. The aim of this study was to examine the capacity of butane-2,3-dionethiosemicarbazone oxime to scavenging different forms of reactive species (RS) *in vitro*, or counteract its formation. Besides, the potential antioxidant and toxic activity of the oxime was assayed both *in vitro* and *ex vivo*. The results indicate a significant H_2O_2, NO and $DPPH^{\bullet}$ radicals scavenging activity at 0.275, 0.5 and 5 μM of oxime, respectively ($p \leq 0.05$). Besides, the oxime exhibited a powerful inhibitory effect on dihydroxybenzoate formation (25 μM) ($p \leq 0.05$) and also decreased deoxyribose degradation induced by Fe^{+2} and via Fenton reaction (0.44 and 0.66 mM, respectively) ($p \leq 0.05$). The oxime showed a significant inhibitory effect on σ-phenantroline reaction with Fe^{+2} (0.4 mM) suggesting a possible interaction between the oxime and iron. A significant decrease in the basal and pro-oxidants induced lipid peroxidation in brain, liver, and kidney of mice was observed both *in vitro* and *ex vivo* ($p \leq 0.05$). In addition, in our *ex vivo* experiments the oxime did not depict significant changes in thiol levels of liver, kidney and brain, as well as did not modify the δ-ALA-D activity in these tissues. Finally, we believe that further studies are necessary to improve our knowledge regarding the exact antioxidant action mechanism and the utility of the oxime as an antioxidant drug.

Key Words: butane-2,3-dionethiosemicarbazone oxime, antioxidant, reactive species, scavenging activity

INTRODUCTION

Reactive species (RS) are commonly generated in normal cellular oxygen metabolism playing some biological roles (Halliwell and Gutteridge, 2006). However, an increase in the RS production over cellular endogenous antioxidant systems capacity of response could result in a condition characterized as oxidative stress (Gutteridge and Halliwell, 1994). Intracellular components could be damaged by an excess of RS generation, which could act as reducing (Halliwell, 2006) or oxidizing (Augusto et al., 2002) agents of biomolecules. In order to remove RS and other secondary products, the cells display several protection and repair mechanisms. The cellular defenses are grouped in enzymatic and non-enzymatic antioxidant systems (Chain et al., 1999).

Some organic intoxication conditions, like the ones caused by pesticides, may induce changes in intracellular metabolism routes leading to an imbalance between the generation of oxidant molecules and the intracellular antioxidant systems (Banerjee et al., 1999). Organophosphates (OPs), which are classical cholinesterase inhibitors used predominately as pesticides, may induce an oxidative stress status in rats (Gultekin et al., 2000; Gupta et al., 2001), mice (Silva et al., 2006 e 2008) and human organisms (Banerjee et al., 1999). Moreover, studies have demonstrated the effects of OPs on lipid peroxidation in rat brain (Fortunato et al., 2006; Verma, 2001) and human erythrocytes (Gultekin et al., 2000). However, the exact mechanism by which OPs induce oxidative damages is not fully understood (Abdollahi et al., 2004).

Oximes are compounds generally used to reverse the AChE inhibition caused by OPs (Worek et al., 2004). Since studies have proposed a link between organophosphate poisoning and changes in human cellular antioxidant systems (Banerjee et al., 1999), researches on compounds acting as cholinesterase reactivators with antioxidant properties may be a good approach for OP intoxication treatments. Conversely, to the best of our knowledge, there are few data concerning possible antioxidant properties of oximes (Puntel et al., 2008 in press).

Thus, the aim of this study was to examine, *in vitro*, the capacity of butane-2,3-dionethiosemicarbazone oxime (Figure 1) to scavenging different forms of RS (such as hydrogen peroxide (H_2O_2), nitric oxide (NO) and 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) as well as counteract hydroxyl radicals ($OH^{\bullet}$) formation (benzoate hydroxylation and deoxyribose degradation). The capacity of butane-2,3-dionethiosemicarbazone oxime as a potential antioxidant agent was assayed using lipid peroxidation (TBARS determination) as endpoint of *in vitro* toxicity both under basal (auto-oxidation of the homogenates) and in the presence of pro-oxidants, including hydrogen peroxide, Fe^{2+}, and malonate (Puntel et al.,

2005a, b). *Ex vivo* experiments were carried out analyzing the effects of butane- 2,3-dionethiosemicarbazone oxime oxime on parameters such as thiol levels, δ-aminolevulinate dehydratase (δ-ALA-D), a sulfhydryl enzyme that can be modified in pro-oxidant situations (Folmer et al., 2003, Perottoni et al., 2005), and basal or pro-oxidants induced lipid peroxidation as markers of some possible toxic or antioxidant effect.

MATERIAL AND METHODS

Chemicals

The oxidant agents such as malonate, hydrogen peroxide and $FeSO_4$ were obtained from local suppliers. σ-phenantroline was obtained from Merck (Darmstadt, Germany). The other reagents used were supplied by Sigma-Aldrich Chemical CO (St. Louis, MO).

Experimental animals

Untreated adult male swiss albino mice were obtained from our own breeding colony. The animals were maintained in an air-conditioned room (20- 25ºC) under a 12 hour light/dark cycle, and with water and food ad libitum. All the experimental procedures performed were conducted according to the guidelines of the Committee of Ethics in Research of the Federal University of Santa Maria, Brazil.

Synthesis of butane- 2,3-dionethiosemicarbazone oxime

The butane-2,3-dione thiosemicarbazone oxime was prepared by mixture of 1 mol diacetylmonoxime with 1 mol of thiosemicarbazide both dissolved in ethanol, made acid by addition of 0.5 mL of acetic acid 0.1M. After three hours of stirring and reflux, pale yellow product was formed, collected by filtration, washed with water and dried in vacuum, yield 70%, mp 225°C. IR: ν = 1595cm^{-1} (C=N), 1296cm^{-1}(C=S), 1087cm^{-1}(O-N), 3251cm^{-1}(N-H), 3420cm^{-1}(O-H). For all experimental procedures the oxime solutions were prepared in ethanol.

In vitro experiments

Assays without tissues in the test tube

Hydrogen peroxide scavenging assay

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

Nitric oxide (NO) scavenging assay

Scavenging of NO was assessed by incubating sodium nitroprusside (SNP) (5 mM, in PBS) with different concentrations of the oxime at 25 °C. After 120 min, 0.5 mL of incubation solution was sampled and mixed with 0.5 mL of Griess reagent (Green et al., 1981). 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 presence of oxime in order to verify its interaction with nitrite, depicted no interference of oxime in 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 oxime as an index of its NO scavenger activity (Marcocci et al., 1994).

DPPH$^{\bullet}$ radical scavenging activity assay

The measurement of the oxime scavenger activity against the radical DPPH$^{\bullet}$ (1,1-diphenyl-2-picrylhydrazyl) was performed in accordance with Choi et al. (2002). Briefly, 85 μM DPPH$^{\bullet}$ was added to a medium containing different oxime concentrations. The mediums were incubated for 30 min at room temperature. The decrease in absorbance measured at 518 nm depicted the scavenger activity of the oxime against DPPH$^{\bullet}$. Ascorbic acid was used as positive control to determine the maximal decrease in DPPH$^{\bullet}$ absorbance (Choi et al., 2002). The values are expressed in percentage of inhibition of DPPH$^{\bullet}$ absorbance in relation to the control values without oxime (ascorbic acid maximal inhibition was considered 100% of inhibition).

Benzoate hydroxylation assay

Benzoate hydroxylation was determined by the reaction between OH$^{\bullet}$ and benzoate producing fluorescent dihydroxybenzoate as described by Gutteridge (1987). OH$^{\bullet}$ were generated by a reaction medium containing H_2O_2 and Fe^{3+}. Briefly, a Fe^{3+}-bipyridyl complex was freshly prepared. Benzoate at 2 mM final concentration was added in a medium containing 0.02 mM of phosphate-buffered saline (0.15 M NaCl/0.1 M sodium phosphate buffer, pH 7.4); 0.16 mM bipyridyl; 0.04 mM $FeCl_3$ and a range of oxime concentrations. The reaction was started by the addition of 0.1 mL of 5 mM H_2O_2 (1 mM final). The tubes were incubated for 1 hour at 37°C. After the incubation period, 0.2 mL of TBA 0.6%, followed by 0.2 mL of TCA 2.8%, were added. The tubes were incubated for 10 min at 100 °C in water bath. The tube contents were extracted with n-butanol and after a brief centrifugation (1,800 x g for 10 min) the upper layer was measured spectrofluorimetrically (Excitation at 305 nm and Emission at 408 nm, and both slit widths used were at 5 nm). The values are expressed in percent of inhibition of dihydroxybenzoate products formation.

Deoxyribose degradation assay

The deoxyribose degradation assay was performed according to Puntel et al. (2005a). Briefly, the reaction medium was prepared containing the following reagents at the final concentrations indicated: oxime (concentrations indicated in the figures), deoxyribose 3 mM, potassium phosphate buffer 0.05 mM, 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 37ºC for 30 min and stopped by the addition of 0.8 mL of trichloroacetic acid (TCA) 2.8% followed by the addition of 0.4 mL of thiobarbituric acid (TBA) 0.6 %. Next, the medium was incubated at 100ºC for 20 min and the absorbance was recorded at 532 nm (Halliwell and Gutteridge, 1981; Gutteridge, 1981). Standard curves of malondialdehyde (MDA) were made in each experiment. The values are expressed as percentage of control values.

Iron chelating properties

In order to examine iron chelating properties butane-2,3-dione thiosemicarbazone oxime we used the σ-phenantroline method (Bucber et al., 1983; Minotti and Aust, 1987) according to Puntel et al. (2005a). The method is based on the reaction of free Fe^{2+} with σ-phenantroline forming a colored complex. First, the mixture containing Fe^{2+} (150 μM) and oxime solutions or its vehicle (ethanol) was allowed to interact for 5 min. Afterwards, a solution of σ-phenantroline was added to the mixture (62.5 μg/mL equivalent to 0.25%). The formation of complexes between Fe^{2+} and oxime was estimated by a decrease in the color reaction at 510 nm when compared to a control tube containing Fe^{2+} and σ-phenantroline alone. The values are expressed in percentage of inhibition in relation to control (Fe^{2+} and σ-phenantroline alone without oxime). The solutions were prepared freshly for each experiment.

Assays with tissues homogenates in the test tube

Tissue preparation

The mice were euthanized by cervical dislocation and the liver, kidney and brain were quickly removed, placed on ice, and homogenized within 10 min in 10 volumes of cold saline 150 mM. The homogenates were centrifuged at 4,000 x g at 4°C for 10 min to yield a low speed supernatant fraction (S1) for each tissue that was used for lipid peroxidation and δ-ALA-D activity assays.

Lipid peroxidation assay

Lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) as described by Ohkawa et al. (1979) in S1 of liver, kidney or brain. Aliquots of S1 (200 μL) were mixed to incubating medium containing Tris-HCl (0.01 mM)

pH 7.4, oxime at indicated concentrations (see figure legends), and pro-oxidant agents when specified, and then incubated at 37ºC for 60 min. The pro-oxidants malonate (mal), iron (Fe^{2+}), hydrogen peroxide (H_2O_2) and sodium nitroprusside (SNP) were added as positive control for lipid peroxidation. The basal or pro-oxidants induced TBARS production was stopped by adding 0.5 mL of acetic acid buffer (pH 3.5), and lipid peroxidation products were measured by the addition of 0.5 mL of TBA 0.6%. Tubes were then incubated in boiling water for 60 min and their contents were extracted with n-butanol before spectrophotometric analysis. TBARS levels were measured at 532 nm using a standard curve of MDA.

***Ex vivo* experiments**

Animals Treatment

Adult male swiss albino mice received a single subcutaneous injection of the oxime dissolved in ethanol in different doses (0.5, 1, 5, 10, 50, 100, 150 or 200 mg/kg). Control animals received ethanol at 5 mL/kg. To determine the potential lethality of butane-2,3-dione thiosemicarbazone oxime, animals were observed for up to 72 h after compound administration. LD_{50} was calculated using "GraphPad Software" (GraphPad software, San Diego, CA). After this period, animals were euthanized by cervical dislocation. The liver, kidney and brain were quickly removed, placed on ice, and homogenized within 10 minutes, in 10 volumes of cold saline 150 mM. The homogenates were centrifuged at 4,000 x g at 4°C for 10 min to yield a low speed supernatant fraction (S1) for each tissue that was used for *ex vivo* analysis of reduced and oxidized glutathione levels, and also for TBARS and δ-ALA-D activity measurement.

Fluorimetric assay of reduced (GSH) and oxidized (GSSG) glutathione

For measurement of GSH and GSSG levels, we used a method previously described by Hissin and Hilf (1976). Briefly, 250 mg of liver, kidney or brain were homogenized in 3.75 mL phosphate-EDTA buffer (pH 8) plus 1 mL HPO_3 (25%). Homogenates were centrifuged at 4°C at 100,000 g for 30 min and the supernatants were separated in two different aliquots of 500µL each for measurement of GSH and GSSG.

For GSH measurement, 500 µL of the supernatant was diluted in 4.5 mL of phosphate-EDTA buffer (pH 8) (sodium phosphate 100 mM and EDTA 5 mM). The final assay mixture (2.0 mL) contained 100 µL of the diluted tissue supernatant, 1.8 mL of phosphate-EDTA buffer and 100 µL of σ-phthalaldehyde (OPA) (1 µg/µL). The mixtures were incubated at room temperature for 15 min and their fluorescent signals were recorded in the luminescence spectrometer at 420 nm of emission and 350 nm of excitation wavelengths.

For measurement of GSSG levels, a 500 µL of the supernatant was incubated at room temperature with 200 µL of n-ethylmaleimide (NEM) (0.04 M) for 30 min in order to prevent the GSH oxidation. To this mixture, 4.3 mL of NaOH (0.1 N) was added. A 100 µL portion of this mixture (NEM-supernatant-NaOH) was taken for measurement of GSSG, using the procedure outlined above for GSH assay, except that NaOH was employed as diluent rather than phosphate-EDTA buffer.

The results were expressed as GSH/GSSG ratio.

δ-ALA-D activity assay

The enzyme activity was assayed according to Sassa method (1982) using S1 of liver, kidney or brain and the assays were conduced as described in the section *in vitro* experiments except that oxime was not added to the reaction medium. Moreover, a set of tubes was assayed simultaneously using a similar incubation medium, except that 2 mM DTT was also added to observe the possible reactivation of the δ-ALA-D activity. The enzyme activity was expressed according to its reactivation index calculated as follows:

$$\frac{(\text{δ-ALA-D activity with DTT} - \text{δ-ALA-D activity without DTT})}{\text{δ-ALA-D activity with DTT}} \times 100\%$$

Lipid peroxidation assay

Lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) as described by Ohkawa et al. (1979) in S1 of liver, kidney or brain. The pro-oxidants iron (Fe^{2+}) and sodium nitroprusside (SNP) were added as positive control for lipid peroxidation. The assays were conduced as described in the section *in vitro* experiments except that oxime was not added to the reaction medium.

Protein determination

The protein content was determined according to Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

Statistical analysis

Data were analyzed by one-way ANOVA, followed by Tukey multiple range test when appropriate. Differences between groups were considered significant when $p \leq 0.05$.

RESULTS

***In vitro* results**

A significant H_2O_2 scavenging activity of oxime was reached at 0.275 µM (figure 2). Likewise the oxime presented a significant NO scavenging activity at 0.5 µM ($p<0.05$)

(Figure 3), and a significant $DPPH^{\bullet}$ scavenging activity at 5 μM ($p<0.05$) (Figure 4). A strong inhibition in dihydroxybenzoate formation was reached at 25 μM of the oxime (Figure 5) ($p<0.05$). The induced Fenton reaction ($Fe^{+2} + H_2O_2$) and Fe^{+2} or H_2O_2 oxidant species, when studied in separate, were able to stimulate deoxyribose degradation (Figure 6). In addition, the oxime decreased deoxyribose degradation induced by Fe^{+2} (0.44 mM) and $Fe^{+2} + H_2O_2$ (0.66 mM) (Figure 6) ($p<0.05$).

Besides, lipid peroxidation in S1 of brain, liver, and kidney induced by malonate, SNP, Fe^{+2}, and H_2O_2 were prevented by oxime at micromolar concentrations (Table 1) ($p<0.05$).On other hand, a significant decrease in σ-phenantroline reaction with Fe^{+2} starting at 0.4 mM of oxime was observed ($p<0.05$) (Table 2). In this context the highest concentration of oxime was able to maintain the depicted profile at significant levels until 72 hours of reaction ($p<0.05$) (Table 2).

***Ex vivo* results**

The oxime treatment did not depict significant changes in liver, kidney and brain glutathione levels (data not show). Moreover δ-ALA-D activity was not modified under these conditions (data not show). On other hand, basal TBARS production was significant decreased by oxime treatment in liver (0.5 to 10 mg/kg) and kidney (0.5 to 10 mg/kg) ($p<0.05$) (Table 3). Besides, SNP induced lipid peroxidation at significant levels, and the oxime effectively prevented the increase in liver (5 and 10 mg/kg) and kidney (1 to 10 mg/kg) ($p<0.05$), but not in brain (Table 3). The Fe^{+2} induced lipid peroxidation was not effectively decreased by the oxime treatment (Table 3).

The LD_{50} calculated was 121 mg/kg of butane-2,3-dione thiosemicarbazone oxime (Figure 7). Furthermore, all groups tested with oxime did not present any loss of body weight (data not shown).

DISCUSSION

In order to elucidate the ability of the 2,3-dione thiosemicarbazone oxime to act toward different RS we tested some radical scavenging assay methods. Our results indicate an important H_2O_2 and NO scavenging activities of 2,3-dione thiosemicarbazone oxime at nanomolar concentrations. Besides, we observed that the oxime can effectively act as scavenger of $OH^{\bullet}$ at low micro molar concentrations, an activity four folds higher than ascorbic acid used as a positive control, in $DPPH^{\bullet}$ assay. Together, these results indicate a strong oxime ability to act against different forms of RS in assays where the direct interaction between oxime and RS was analyzed.

An important mechanism to accelerate the intracellular components dysfunction is caused by Fe^{2+} + H_2O_2 reaction that can lead to $OH^{\bullet}$ formation (Halliwell and Gutteridge, 1984). A classic model to evaluate the interaction between different agents and *in vitro* $OH^{\bullet}$ formed via Fe^{2+} + H_2O_2 reaction is the deoxyribose degradation assay (Halliwell and Gutteridge, 1981; Gutteridge, 1981). Our results indicate that the oxime effectively decreased deoxyribose degradation induced by Fe^{2+} and Fe^{2+} + H_2O_2 at micro molar concentrations, suggesting an important effect against Fe^{2+} and $OH^{\bullet}$ formed via Fe^{2+} + H_2O_2 reaction.

Besides, another *in vitro* mechanism to evaluate the $OH^{\bullet}$ formation is through benzoate hydroxylation assay where $OH^{\bullet}$ are formed via Fe^{3+} + H_2O_2 reaction (Gutteridge, 1987). In such conditions $OH^{\bullet}$ are formed at low levels in comparison to the Fe^{2+} + H_2O_2 reaction since the Fe^{3+} react with bipyridyl and form a pyrrolic complex limiting Fe^{3+} + H_2O_2 reaction (Gutteridge, 1987). Our data indicate that the oxime practically abolished the dihydroxybenzoate formation at low micro molar concentrations. The lower oxime concentration used to limit the benzoate hydroxylation in comparison to the concentrations used to decrease the deoxyribose degradation could be due to the lower levels of $OH^{\bullet}$ formation.

According to our results we propose that besides of the oxime ability to directly neutralize the formed $OH^{\bullet}$ ($DPPH^{\bullet}$ assay) it can also counteract the $OH^{\bullet}$ formation via Fe^{2+} + H_2O_2 reaction and also Fe^{3+} + H_2O_2 reaction. Besides, our *in vitro* experiments searching for possible oxime-iron interaction reveal a significant capacity of butane-2,3-dione thiosemicarbazone oxime to interact with Fe^{2+}, since the oxime was able to decrease the σ-phenantroline reaction with Fe^{2+}. It is well established that molecules containing sulfur ligands stabilize Fe^{2+} acting as chelators, and therefore decrease the availability of iron to react with other molecules (Miller et al., 1990). This data could be used as a explanation to the results obtained when oxime was incubated with Fe^{2+} since the butane-2,3-dione thiosemicarbazone oxime present a sulfur atom in its structure (Figure 1).

Actually is well recognized that iron is essential in numerous biochemical processes, including oxygen transport, cellular respiration and metabolism, drug metabolism, and DNA synthesis (Welch et al., 2002). However, iron is also recognized to be integrally involved in many biochemical oxidation reactions (Welch et al., 2002), which are on the basis of pathological disorders like neurodegeneration (Molina-Holgado, 2007) and atherosclerosis (Ong and Halliwell, 2004). Besides, compounds that interact with iron and thus block its oxidative reactions with biological components could be used as potential antioxidant agents (Miller et al., 1990; Ryan et al., 1993; Welch et al., 2002). Thus, we believe that the butane-

2,3-dione thiosemicarbazone oxime could be used as a further antioxidant agent in stressing conditions involving the oxidative damage induced by iron.

When test in a *in vitro* medium containing homogenate of tissues (brain, liver or kidney) the butane-2,3-dione thiosemicarbazone oxime affect the basal and pro-oxidant (malonate, Fe^{2+}, H_2O_2 or SNP) induced lipid peroxidation at micromolar concentrations. Taken together our results point to a significant antioxidant capacity of the oxime against the basal and the pro-oxidants induced TBARS formation.

On other hand, *ex vivo* results demonstrated that oxime was also able to decrease the basal and SNP-induced TBARS formation in liver and kidney. A possible explanation for these results could be the NO radical scavenger activity depicted by the oxime, since SNP is well recognized as a classical NO donor in presence of reducing agents (Bates et al., 1991). The NO released from SNP molecules decomposition could react with intracellular superoxide anions (O_2^-) and to form peroxynitrite ($ONOO^-$) which is a damaging oxidant involved in tissues damage (Villa et al., 1994). However, the decomposition of SNP molecules also release pentacyanoferrate complex (Rao et al., 1991; Roncaroli et al., 2005; Bates et al., 1991) which could lead to oxidative reactions involved in RS generation (Welch et al., 2002). Although our *in vitro* results suggest a capacity of oxime to interact with iron we did not observed effects on *ex vivo* lipid peroxidation induced by Fe^{2+} in the tested tissues. Taken together, these results indicate NO as the main responsible for the oxidative damage induced by SNP, since the oxime did not decrease the lipid peroxidation induced by Fe^{2+}. A possible explanation for the absence of effect on *ex vivo* lipid peroxidation induced by Fe^{2+} could be a metabolic process that involves the oxime, and thus alter the oxime-Fe^{2+}interaction capacity. On other hand, the absence of oxime effects in brain tissue may be due to its low capacity to cross the brain-blood barrier (BBB). Previews studies have reported that the mean BBB penetration of pralidoxime (2-PAM), a common clinically used oxime, is approximately 10% (Sakurada et al., 2003).

Furthermore, according the toxicological parameters analyzed in *ex vivo* experiments we observed that the oxime depicted no one significant change in GSH/GSSG levels (data not shown). In the same way were not observed significant changes in *ex vivo* δ-ALA-D activity in all tissues tested (data not shown). According to our results we suggest that the treatment with butane-2,3-dione thiosemicarbazone oxime depicted low toxicity signals. Thus, we believe that this oxime could be used as a safe drug for further studies. Finally, LD_{50} value was similar to observed with other oximes like pralidoxime and obidoxime (Arena, 1979).

CONCLUSION

In conclusion, the present study demonstrated an *in vitro* antioxidant activity of butane-2,3-dione thiosemicarbazone oxime in scavenging different forms of RS like hydroxyl radicals, nitric oxide radicals, and hydrogen peroxide as well as counteract its formation (benzoate hydroxylation and deoxyribose degradation). Our *in vitro* experiments performed in presence of homogenates tissues revealed that the oxime was able to effectively counteract the lipid peroxidation induced by different oxidant agents. Furthermore, *ex vivo* results point to the maintenance of antioxidant capacity of oxime in liver and kidney since that occurred a decrease in the basal and SNP- induced TBARS production. Taken together these results indicate an *in vitro* and *ex vivo* antioxidant activity of the oxime possibly due to its scavenger activity toward different RS and also related with its metal chelating properties. Finally, we believe that further studies are necessary to improve the knowledge regarding its exact antioxidant action mechanism.

AKNOWLEDGMENTS

Work Supported by the FINEP research grant “Rede Instituto Brasileiro de Neurociência (IBN-Net)” # 01.06.0842-00. P.G. receives a fellowship by CNPq/PIBIC/UFSM. A.P. receives a fellowship by BIC/FAPERGS. G.O.P. receives a fellowship by CAPES. Additional support giving by CNPq, FAPERGS and CAPES.

REFERENCES

Abdollahi, M., Ranjbar, A., Shadnia, S., Nikfar, S., Rezaie, A., 2004. Pesticides and oxidative stress: a review. Medical Science Monitor 10(6), 141-147.

Arena, J.M., 1979. Poisoning, Toxicology, Symptoms, Treatments, forth edition, Springfield Ill, Charles C.Thomas, p. 133.

Augusto, O., Bonini, M.G., Amanso, A.M., Linares, E., Santos, C.C., De Menezes, S.L, 2002. Nitrogen dioxide and carbonate radical anion: two emerging radicals in biology. Free Radicals Biology and Medicine. 32, 841–859.

Banerjee, B.D., Seth, V., Bhattacharya, A., Pasha, S.T., Chakraborty, A.K., 1999. Biochemical effects of some pesticides on lipid peroxidation and free-radical scavengers. Toxicology Letters. 107, 33–47.

Bates, J.N., Baker, M.T., Guerra, Jr.R., Harrison, D.G., 1991. Nitric oxide generation from nitroprusside by vascular tissue. Evidence that reduction of the nitroprusside anion and cyanide loss is required. Biochemical Pharmacology 42, S157-S165.

Bucber, J.R., Tien, M., Morehouse, L.A., Aust, S.D, 1983. Redox cycling and lipid peroxidation: The central role of iron chelates. Fundamental and Applied Toxicology 3, 222-226.

Chain, A.C., Chow, C.K., Chiu, D, 1999. Interaction of Antioxidants and Their Implication in Genetic Anemia. Proceedings of the Society for Experimental Biology and Medicine. 274-282.

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

Folmer, V., Soares, J.C.M., Gabriel, D., Rocha, J.B.T, 2003. A high fat diet inhibits δ-aminolevulinate dehydratase and increases lipid peroxidation in mice (Mus musculus). The Journal of Nutrition 133(7), 2165-2170.

Fortunato, J.J., Feier, G., Vitali, A.M., Petronilho, F.C., Dal-Pizzol, F., Quevedo, J., 2006. Malathion induced oxidative stress in rat brain regions. Neurochemical Research 31, 671-678.

Green, L.C., Wanger, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R, 1981. Analysis of nitrate, nitrite, [^{15}N]nitrate in biological fluids. Analytical Biochemistry 126, 131–138.

Gultekin, F., Ozturk, M., Akdogan, M., 2000. The effect of organophosphate insecticide chlorpyrifos-ethyl on lipid peroxidation and antioxidant enzymes (*in vitro*). Archives of Toxicology. 74, 533-568.

Gupta, R.C., Milatovic D., Dettbarn, W.D., 2001. Depletion of energy metabolites following acetylcholinesterase inhibitor-induced sattus epilepticus: Protection by antioxidants. Neurotoxicology 22, 271-282.

Gutteridge, J.M.C, 1987. Ferrous-salt-promoted damage to deoxyribose and benzoate. The increased effectiveness of hydroxyl-radical scavengers in the presence of EDTA. Biochemical Journal, 243(3), 709–714.

Gutteridge, J.M.C., 1981. Thiobarbituric acid-reactivity following iron-dependent free-radical damage to amino acids and carbohydrates. FEBS Letters. 128, 343-346.

Gutteridge, J. M. C., Halliwell, B., 1994. Antioxidants in nutrition, Health and Disease. New York: Oxford University Press. 111–123.

Halliwell, B., 2006. Oxidative stress and neurodegeneration: Were are we now? Journal of Neurochemistry. 97, 1634–1658.

Halliwell, B., Gutteridge, J.M.C., 1981. Formation of a thiobarbituric-acid-reactive substance from deoxyribose in the presence of iron salts: The role of superoxide and hydroxyl radicals. FEBS Letters. 128, 347-352.

Halliwell, B., Gutteridge, J.M.C., 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochemical Journal 219, 1–14.

Halliwell, B., Gutteridge, J.M.C., 2006. Free Radicals in Biology and Medicine, 4th edn, Oxford University Press, Oxford.

Hissin, P.J., Hilf, R., 1976. A fluorimetric method for determination of oxidized and reduced glutathione in tissues. Analytical Biochemistry 74, 214–226.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. Biological Chemistry. 193, 265–275;

Marcocci, L., Maguire, J.J., Droy-Lefaix, M.T., Packer, L., 1994. The nitric oxide-scavenging properties of Ginkgo biloba extract EGb 761. Biochemical and Biophysical Research Communications, 201, 748–755.

Miller, D.M., Buettner, G.R., Aust, S.D., 1990. Transition metals as catalysts of "autoxidation" reactions. Free Radicals Biology & Medicine 8, 95–108.

Minotti, G., Aust, S.D., 1987. An investigation into the mechanism of citrate-Fe^{2+}-dependent lipid peroxidation. Free Radical Biology and Medicine 3, 379- 387.

Molina-Holgado, F., Hider, R.C., Gaeta, A., Williams, R., Francis, P., 2007. Metal ions and neurodegeneration. Biometals 20, 639-654.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry 95, 351- 358.

Ong, W.Y., Halliwell, B., 2004. Iron, Atherosclerosis, and Neurodegeneration: A Key Role for Cholesterol in Promoting Iron-Dependent Oxidative Damage? Annals of the New York Academy of Sciences, 1012, 51-64.

Perottoni, J., Meotti, F.C., Folmer, V., Pivetta, L., Nogueira, C.W., Zeni, G., Rocha, J.B.T., 2005. Ebselen and diphenyl diselenide do not change the inhibitory effect of lead acetate on delta-aminolevulinate dehidratase. Environmental Toxicology and Pharmacology 19, 239–248.

Puntel, G.O., Gubert, P., Peres, G.L., Bresolin, L., Rocha, J.B.T., Pereira, M.E., Carratu, V.S., Soares, F.A.A., 2008. Antioxidant properties of oxime 3-(phenylhydrazone) butan-2-one. Arch. Tox. In press.

Puntel, R.L., Nogueira, C.W., Rocha, J.B.T., 2005a. Krebs cycle intermediates modulate thiobarbituric acid reactive species (TBARS) production in rat brain *in vitro*. Neurochemical Research 30, 225–235.

Puntel, R.L., Nogueira, C.W., Rocha, J.B.T., 2005b. N-methyl-D-aspartate receptors are involved in the quinolinic acid, but not in the malonate pro-oxidative activity *in vitro*. Neurochemical Research 30, 417–424.

Rao, D.N., Elguindi, S., O'Brien, P.J., 1991. Reductive metabolism of nitroprusside in rat hepatocytes and human erythrocytes, Archives of biochemistry and biophysics, 286, 30–37.

Rios, J.C., Repetto, G., Galleguillos, I., Jos, A., Peso, A.D., Repetto, M., 2005. High concentrations of pralidoxime are needed for the adequate reactivation of human erythrocyte acetylcholinesterase inhibited by dimethoate *in vitro*. Toxicology *In Vitro* 19(7), 893-897.

Roncaroli, F. Roncaroli, Eldik, R.V., Olabe, J.A., 2005. Release of NO from reduced nitroprusside ion. Iron-dinitrosyl formation and NO-disproportionation reactions. Inorganic Chemistry 44, 2781–2790.

Ryan, T.P., Miller, D.M., Aust, S.D., 1993. The role of metals in the enzymatic and nonenzymatic oxidation of epinephrine. Journal of Biochemical Toxicology 8, 33–39.

Sakurada, K., Matsubara, K., Shimizu, K., Shiono, H., Seto, Y., Tsuge, K.,Yoshino, M., Sakai, I., Mukoyama, H., Takatori, T., 2003. Pralidoxime iodide (2-PAM) penetrates across the blood–brain barrier. Neurochemical Research 28, 1401–1407.

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

Silva, A.P., Meotti, F.C, Santos, A.R.S., Farina, M., 2006. Lactational exposure to malathion inhibits brain acetylcholinesterase in mice. Neurotoxicology, 27, 1101-1105.

Silva, A.P., Farina, M., Franco, J.L., Dafre, A.L., Kassa, J., Kuca, C., 2008. Temporal effects of newly developed oximes (K027, K048) on malathion-induced acetylcholinesterase inhibition and lipid peroxidation in mouse prefrontal cortex. Neurotoxicology, 29, 184-189.

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.A., 1988. Simple technique for quantification of low levels of DNA damage in individual cells. Experimental Cell Research 175, 184–191.

Verma R.S., 2001. Chlorpyrifos-induced alterations in levels of thiobarbituric acid reactive substances and glutathione in rat brain. Indian Journal of Experimental Biology 39, 174–77.

Villa, L.M., Salas, E., Darley-Usmar, V.M. Radomski, M.W., Moncada, S., 1994. Peroxynitrite induces both vasodilatation and impaired vascular relaxation in the isolated perfused rat heart. Proceedings of the National Academy of Sciences of the United States of America 91, 12383-12387.

Welch, K.D., Davis, T.Z., Van Eden, M.E., Aust, S.D., 2002. Deleterious iron-mediated oxidation of biomolecules. Free Radicals Biology & Medicine, 32(7), 577-583.

Worek, F., Thiermann, H., Szinicz, L., Eyer, P., 2004. Kinetic analysis of interactions between human acetylcholineserase, structurally different organophosphorus compounds and oximes. Biochemical Pharmacology 68, 2237–2248.

Figure 1:

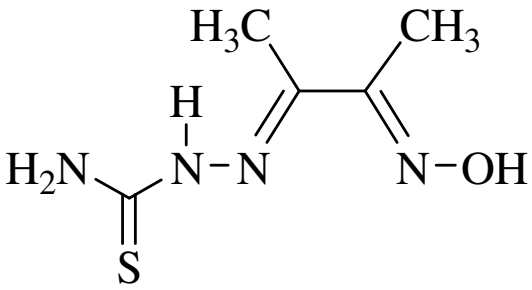

Figure 1: **Chemical structure of butane-2,3-dione thiosemicarbazone oxime**.

Figure 2:

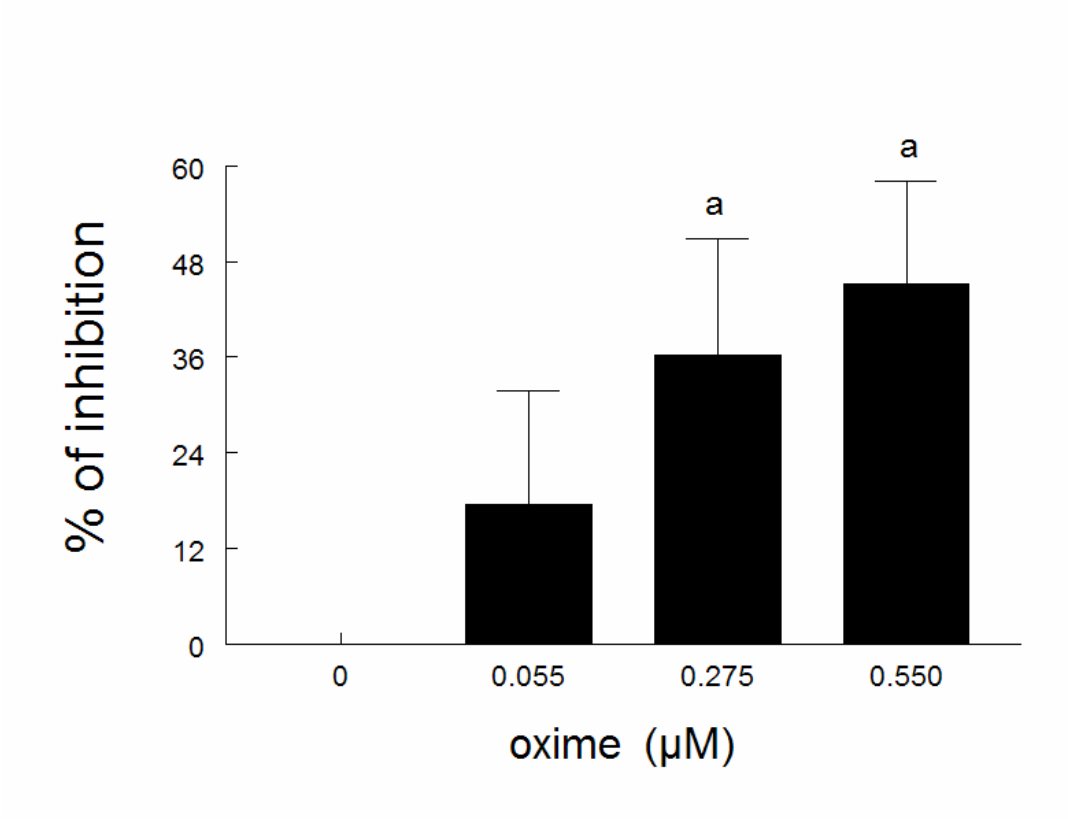

Figure 2: **Effect of oxime on hydrogen peroxide (H_2O_2) scavenging assay**: The values are expressed in percent of inhibition of luminol oxidation by H_2O_2 in relation to control without oxime. The mean control value is 3.510.971 ± 592.490 cpm. Data are expressed as mean ± SEM (n=6). (^{a}) indicate $p<0.05$ from respective control without oxime by Tukey multiple range test's.

Figure 3:

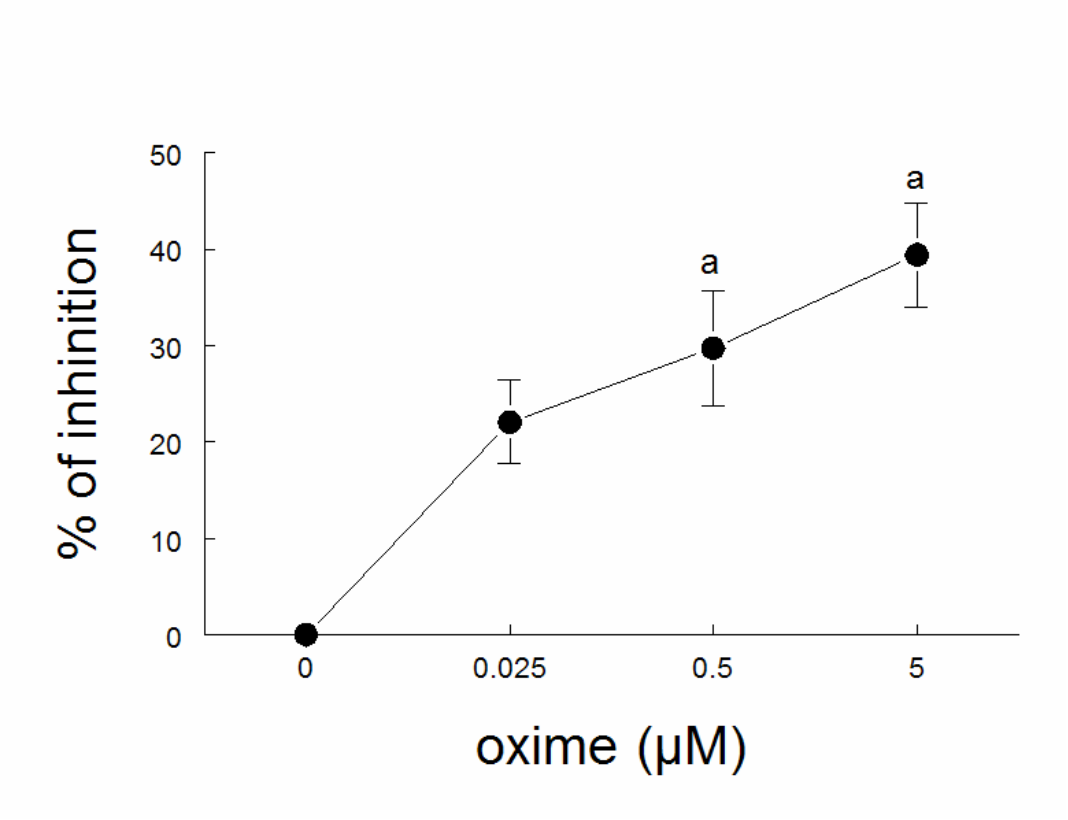

Figure 3: **Effects of oxime on nitric oxide (NO) scavenging assay**. The values are expressed in percentage of inhibition in relation to control without oxime. The mean control value is 18.4±1.6 µM of nitrite. Data are presented as mean ± SEM (n=3). ([a]) indicate $p<0.05$ from respective control without oxime by Tukey multiple range test's.

Figure 4:

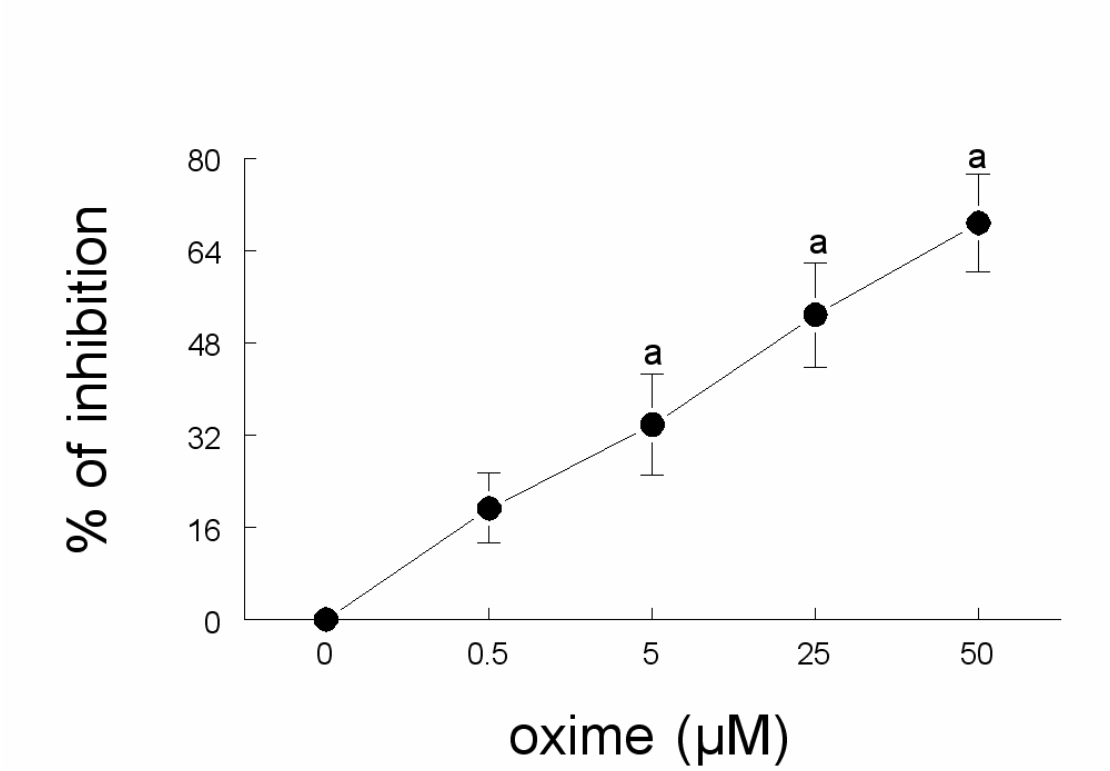

Figure 4: **DPPH• radical scavenging activity of oxime**. The values are expressed in percentage of inhibition in relation to control without oxime or ascorbic acid. The mean control value is 0.658±0.02 absorbance (518 nm). Data are presented as mean ± SEM (n=3). ([a]) indicate $p<0.05$ from respective control without oxime by Tukey multiple range test's.

Figure 5:

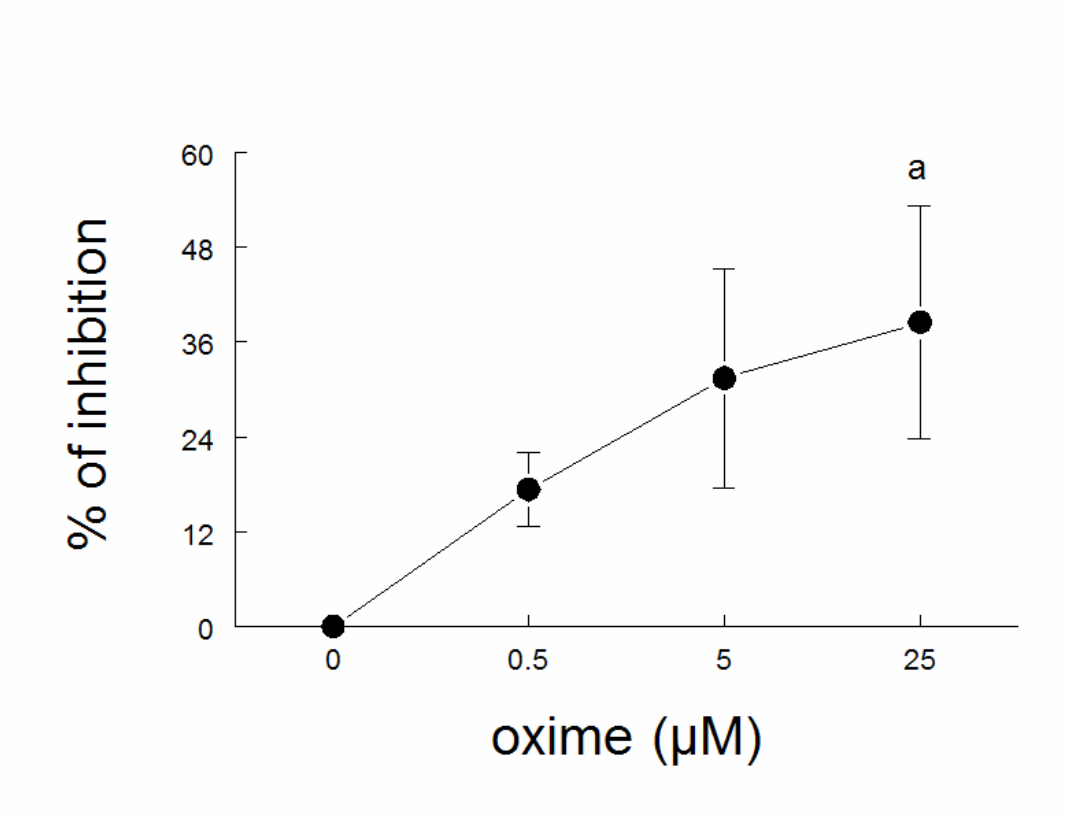

Figure 5: **Effects of oxime on benzoate hydroxylation**. The values are expressed in percent of inhibition of dihydroxybenzoates products formation. The mean basal control value is 183.34 ± 3.64 flourescence units. Data are presented as mean ± SEM (n=3). ([a]) indicate $p<0.05$ from respective control without oxime by Tukey multiple range test's.

Figure 6:

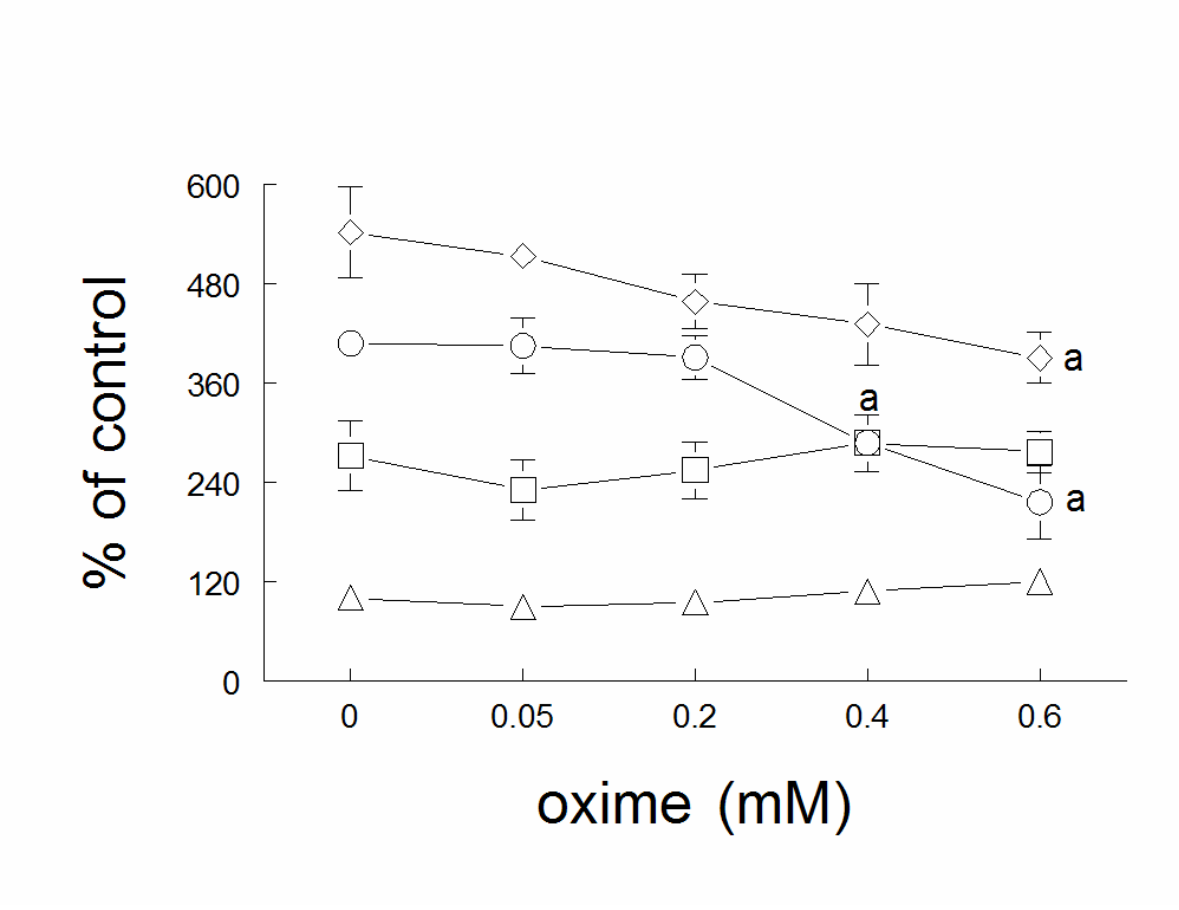

Figure 6: **Effects of oxime on deoxyribose degradation**. The (-Δ-) indicates basal conditions, (-□-) hydrogen peroxide at 500 µM, (-○-) Fe^{2+} at 5 µM, and (-◊-) Fe^{2+} 5 µM plus hydrogen peroxide 500 µM conditions. The values are expressed as percentage of control values. The mean control value is 0.548±0.003 µM MDA/g of deoxyribose. Data are presented as mean ± SEM (n=3). (^{a}) indicate $p<0.05$ from respective control without oxime by Tukey multiple range test's.

Figure 7:

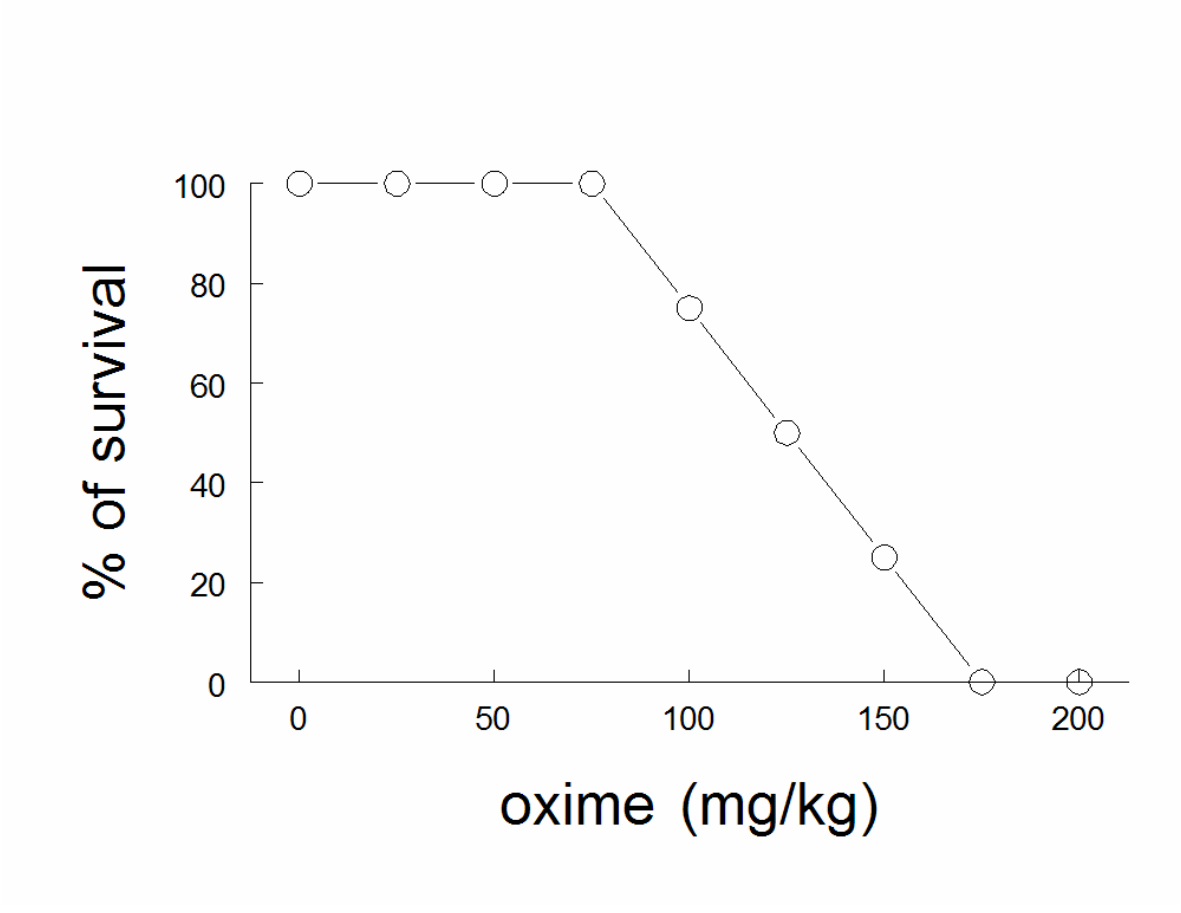

Figure 7: **Effects of oxime administration on mice survival**. Potential lethality of subcutaneous administration of butane-2,3-dionethiosemicarbazone oxime in mice. The data are expressed in percentage of survival (n=6).

TABLE 1: *In vitro* TBARS production:

	LIVER					KIDNEY					BRAIN				
	BAS	**Mal**	**Fe^{2+}**	**H_2O_2**	**SNP**	**BAS**	**Mal**	**Fe^{2+}**	**H_2O_2**	**SNP**	**BAS**	**Mal**	**Fe^{2+}**	**H_2O_2**	**SNP**
Control	3.4±0.7	5.3±0.3	5.2±0.1	6.1±0.6	6.0±0.3	3.7±0.1	6.1±0.9	5.7±0.1	6.6±1.0	8.2±0.2	10.2±0.9	18.7±1.0	28.7±0.6	22.2±0.9	18.4±1.5
50 µM	2.7±0.3	4.5±0.9	5.1±0.1	4.9±0.9 [a]	6.2±0.2	3.0±0.5	6.9±1.2	5.6±0.1	5.9±0.9	7.8±0.2	12.0±1.1	18.5±1.0	30.3±0.5	25.1±1.0	17.7±2.6
200 µM	1.7±0.4 [a]	2.7±0.6 [a]	2.3±0.4 [a]	3.2±0.9 [a]	4.1±0.9 [a]	2.6±0.7 [a]	4.0±0.8 [a]	4.2±0.1 [a]	4.4±1.1 [a]	7.5±0.6	6.8±0.5	14.8±0.5	24.0±0.4	19.7±0.6	15.8±2.4
400 µM	1.5±0.2 [a]	2.8±0.6 [a]	1.7±0.2 [a]	3.4±0.9 [a]	3.7±0.4 [a]	1.7±0.1 [a]	3.5±0.6 [a]	2.8±0.1 [a]	3.6±1.0 [a]	4.1±0.9 [a]	2.5±0.5 [a]	3.8±0.5 [a]	8.9±0.4 [a]	8.2±0.5 [a]	7.4±0.1 [a]
600 µM	1.4±0.1 [a]	2.0±0.3 [a]	1.4±0.1 [a]	3.1±0.1 [a]	3.8±0.8 [a]	1.6±0.1 [a]	3.4±0.6 [a]	1.7±0.1 [a]	2.8±0.2 [a]	3.2±0.6 [a]	2.4±0.6 [a]	2.4±0.6 [a]	3.8±0.5 [a]	3.9±0.4 [a]	5.0±0.1 [a]

Table 1: **Effects of butane-2,3-dionethiosemicarbazone oxime on basal (bas) or pro-oxidants induced TBARS production in low-speed supernatant (S1) from brain, liver and kidney**. The pro-oxidants were malonate (mal) at 4mM, iron (Fe^{2+}) at 5µM, hydrogen peroxide (H_2O_2) at 500µM, or sodium nitroprusside (SNP) at 5 µM. TBARS levels are expressed as nmol of MDA/mg of protein. Data are presented as mean ± SEM (n=3). ([a]) indicate $p<0.05$ from respective control without oxime by Tukey multiple range test's.

TABLE 2: Iron chelating properties of oxime:

	0 hours	1 hour	2 hours	12 hours	24 hours	48 hours	72 hours
Control	0.0 ± 0.0	1.5 ± 0.5	1.0 ± 0.15	0.7 ± 0.2	1.5 ± 0.5	5.0 ± 2.5	2.5 ± 0.5
50 µM	8.5 ± 7.5	7.5 ± 7.0	8.5 ± 6.0	8.5 ± 7.5	5.0 ± 6.5	1.5 ± 6.0	1.2 ± 0.2
200 µM	20 ± 10.5	18.0 ± 8.0	17.5 ± 7.5	11.0 ± 8.5	5.1 ± 10.8	1.5 ± 6.0	1.2 ± 0.0
400 µM	45.0 ± 7.0 [a]	43.0 ± 5.5 [a]	42.5 ± 4.0 [a]	33.0 ± 6.0 [a]	13.5 ± 9.5	24.0 ± 5.0	6.0 ± 1.0
600 µM	60.0 ± 11.0 [a]	68.0 ± 3.0 [a]	67.5 ± 2.5 [a]	63.7 ± 3.7 [a]	62.5 ± 4.5 [a]	57.5 ± 2.5 [a]	54.0 ± 1.5 [a]

Table 2: **Effects of butane-2,3-dionethiosemicarbazone oxime on iron chelating assay**. The values are expressed in percent of inhibition in relation to control without oxime. The mean control value is 1.171 ± 0.065 units of absorbance (510 nm). Data are presented as mean ± SEM (n=3). ([a]) indicate $p<0.05$ from respective control without oxime by Tukey multiple range test's.

TABLE 3: *Ex vivo* TBARS production:

	LIVER			KIDNEY			BRAIN		
	Basal	**Fe^{2+}**	**SNP**	**Basal**	**Fe^{2+}**	**SNP**	**Basal**	**Fe^{2+}**	**SNP**
Control	2.7±0.1	9.9±0.98	9.0± 0.1	2.5±0.1	6.8±1.4	6.5±0.2	11.6±0.1	17.2±0.4	16.7±1.0
0.5 mg/kg	1.6±0.1 [a]	8.7± 1.4	8.7±0.2	1.7±0.2 [a]	5.4±0.9	5.5±0.2	12.4±0.1	16.5±1.9	15.9±3.8
1 mg/kg	1.4±0.1 [a]	8.5±1.4	8.6± 0.2	1.9±0.2 [a]	5.1±0.9	5.2±0.1 [a]	13.5±0.1	16.9±1.6	17.3±2.5
5 mg/kg	1.5±0.2 [a]	7.7±1.4	7.5± 0.3 [a]	1.8±0.1 [a]	5.1±0.5	5.2±0.3 [a]	12.6±1.9	16.8±0.6	16.6±4.2
10 mg/kg	1.6±0.3 [a]	7.2±2.1	7.2± 1.4 [a]	1.3±0.2 [a]	5.2±1.0	4.4±0.8 [a]	11.9±0.2	16.7±1.2	16.3±0.9

Table 3: **Effect of the treatment with butane-2,3-dionethiosemicarbazone oxime on low-speed supernatant (S1) from liver, kidney and brain TBARS production**. Sodium nitroprusside (SNP) at 5 μM and iron (Fe^{2+}) at 5 μM were used as pro-oxidants to induce TBARS production. TBARS are expressed as nmol of MDA per mg of protein. Data are presented as mean ± SEM (n=3). ([a]) indicate $p<0.05$ from respective control without oxime by Tukey multiple range test's.

4. DISCUSSÃO

Os resultados apresentados no **Manuscrito 1** sugerem que a atividade antioxidante da oxima 3-(fenil hidrazona) butano-2-ona (Figura 1, Manuscrito 1) está possivelmente relacionada a metabolização das suas moléculas em presença de constituintes celulares. Esta conclusão é baseada nos resultados *in vitro*, os quais mostram que a oxima determinou uma diminuição significativa nos níveis de TBARS basais ou induzidos por diferentes agentes pro-oxidantes em preparações de cérebro de camundongos (Figura 6A, Manuscrito 1). É importante salientar que estes resultados foram obtidos com concentrações nanomolares da oxima, as quais não foram capazes de diminuir os níveis de TBARS basais ou induzidos por agentes pro-oxidantes em preparações contendo apenas lipídios (Figura 6B, Manuscrito 1) ou desoxirribose (Figura 2, Manuscrito 1). Além disso, a hipótese da metabolização das moléculas da oxima na gênese de suas propriedades antioxidantes é corroborada pelo seu efeito em diminuir os níveis basais de TBARS das preparações de cérebro de camundongos, e não apenas em limitar os efeitos oxidantes dos diferentes indutores de dano oxidativo empregados (Figura 6A, Manuscrito 1).

Outra importante conclusão no estudo da origem das propriedades antioxidantes da oxima 3-(fenil hidrazona) butano-2-ona é o fato de que esta não interferiu na reação Fe^{2+} + H_2O_2 induzida experimentalmente. Esta conclusão é baseada na ausência de efeitos da oxima na degradação da desoxirribose (Figura 2, Manuscrito 1), na indução de TBARS em lipídios (Figura 6B, Manuscrito 1), e na reação de hidroxilação do benzoato (Figura 2, Manuscrito 1). Os nossos resultados sugerem que a ausência de efeitos da oxima na reação Fe^{2+} + H_2O_2 possivelmente está relacionada à sua incapacidade de interação íons Fe^{2+}. Esta hipótese é corroborada pelo fato de que a oxima não interferiu de maneira significativa na reação da σ-fenantrolina com íons Fe^{+2} (dado não apresentado). Desta forma, sugerimos que a atividade antioxidante da oxima não está correlacionada com a sua capacidade de interagir com de íons Fe^{2+}.

Além disso, os resultados obtidos sugerem que a capacidade da oxima 3-(fenil hidrazona) butano-2-ona em neutralizar o radical $DPPH^{\bullet}$ e a formação do radical NO não está diretamente relacionada à origem de suas propriedades antioxidantes. Esta conclusão baseia-se no fato de que concentrações micromolares da oxima foram necessárias para

neutralizar de maneira significativa os radicais $DPPH^{\bullet}$ (Figura 4, Manuscrito1), enquanto os efeitos observados na redução dos níveis de TBARS em preparações de cérebro de camundongos foram observados em concentrações nano molares (Figura 6A, Manuscrito 1). O radical $DPPH^{\bullet}$ é uma molécula estável frequentemente utilizada para investigar a capacidade de um composto neutralizar os radicais $OH^{\bullet}$ (Choi e cols., 2002). Desta forma, a análise da capacidade da oxima em neutralizar o radical $DPPH^{\bullet}$ esta diretamente relacionada a sua capacidade de neutralizar os radicais $OH^{\bullet}$. Por sua vez, a capacidade de neutralizar a formação de radicais NO foi observada em concentrações nanomolares da oxima (Figura 5, Manuscrito 1), porém ligeiramente maiores do que as efetivas na redução dos níveis de TBARS (Figura 6A, Manuscrito 1). A partir da análise destes resultados podemos inferir que a alta atividade antioxidante não pode ser diretamente atribuída a sua habilidade de neutralizar ERs como os radicais $OH^{\bullet}$ e NO, corroborando a hipótese de formação de metabólitos ativos com alta capacidade antioxidante.

O estudo dos efeitos tóxicos da oxima 3-(fenil hidrazona) butano-2-ona, a partir dos experimentos realizados *in vivo* e *ex* vivo, sugerem que a oxima apresentou uma toxicidade pequena em camundongos. Esta conclusão baseia-se no fato de que a dose letal da oxima para cinqüenta por cento da amostra (LD_{50}) foi maior que 500 mg/kg quando administrada subcutaneamente em camundongos (dado não apresentado). Os resultados das análises *ex vivo*, por sua vez, mostram que o tratamento com a oxima não determinou alterações significativas nos níveis de tióis não protéicos (NPSH) (Figura 8, Manuscrito 1) e na atividade da enzima δ-ALA-D (Figura 10, Manuscrito 1) em preparações de cérebro de camundongos. Além disso, a atividade *in vitro* da enzima δ-ALA-D não foi significativamente alterada pela ação da oxima (Figura 7, Manuscrito 1). Desta forma, os resultados obtidos indicam que a oxima apresentou uma baixa toxicidade uma vez que a determinação dos níveis de NPSH e da atividade da enzima δ-ALA-D, utilizados como parâmetro de dano oxidativo, não foram significativamente alterados pela ação da oxima tanto nas análises realizadas *in vitro* (atividade da enzima δ-ALA-D apenas) quanto nas realizadas *ex vivo* após o tratamento dos camundongos com a oxima.

Por fim, sugerimos que a meia-vida plasmática curta da oxima 3-(fenil hidrazona) butano-2-ona pode ser considerada uma explicação para a ausência de efeitos da oxima nos níveis de TBARS basais ou induzidos por agentes pro-oxidantes nas análises *ex vivo* em preparações de cérebro de camundongos (Figura 9, Manuscrito 1). É importante salientar que as análises *ex vivo* foram realizadas 72 horas após a administração subcutânea da oxima, e que

a meia-vida plasmática relativamente curta de algumas oximas como a pralidoxima (75 min) já estão bem relatadas na literatura (Howland e Aaron, 1999).

Os resultados apresentados no **manuscrito 2** indicam uma importante atividade antioxidante da oxima butano-2,3-dionatiosemicarbazona (Figura 1, Manuscrito 2) ao diminuir significativamente os níveis de TBARS basais e induzidos por agentes pro-oxidantes em diferentes tecidos de camundongos em concentrações micromolares da oxima (Tabela 1, Manuscrito 2). Um dos possíveis mecanismos envolvidos na gênese das propriedades antioxidantes da oxima refere-se a sua capacidade de neutralizar diferentes formas de ERs como o H_2O_2, e o radical $DPPH^{\bullet}$, além de neutralizar a formação do radical NO. Esta conclusão está baseada nos resultados obtidos onde é possível observar que a espécie reativa H_2O_2 (Figura 2, Manuscrito 2), e a formação do radical NO (Figura 3, Manuscrito 2) foram neutralizadas em concentrações nano molares da oxima. Por sua vez, a capacidade de neutralizar os radicais $DPPH^{\bullet}$ foi observada em concentrações micromolares da oxima (Figura 4, Manuscrito 2), sugerindo que a oxima pode também atuar neutralizando os radicais $OH^{\bullet}$.

Outra importante conclusão obtida neste estudo refere-se ao fato de que a oxima butano-2,3-dionatiosemicarbazona, além da capacidade de neutralizar diretamente os radicais $OH^{\bullet}$, interfere significativamente na formação destes radicais. Esta conclusão está baseada na sua capacidade de limitar a reação entre $Fe^{2+} + H_2O_2$, e também a reação $Fe^{3+} + H_2O_2$, as quais são responsáveis pela geração de radicais $OH^{\bullet}$. Os resultados mostram que a oxima limitou a formação de radicais $OH^{\bullet}$, uma vez que diminuiu significativamente a hidroxilação do benzoato, a partir da reação $Fe^{3+} + H_2O_2$, em concentrações micro molares (Figura 5, Manuscrito 2). Além disso, a hipótese da oxima limitar a formação de radicais $OH^{\bullet}$ foi corroborada pela sua capacidade de diminuir a degradação da desoxirribose induzida pela reação $Fe^{2+} + H_2O_2$ em concentrações micro molares (Figura 6, Manuscrito 2). É importante ressaltar que concentrações relativamente menores de oxima foram necessárias para limitar a hidroxilação do benzoato de maneira significativa, em comparação com as concentrações necessárias para limitar a degradação da desoxirribose. Uma possível explicação para este resultado é o fato de que na reação entre $Fe^{3+} + H_2O_2$, induzida experimentalmente pela reação entre o complexo cloreto de ferro ($FeCl_3$)/bipiridil e as moléculas de H_2O_2 no ensaio experimental da hidroxilação do benzoato, ocorre uma menor formação de radicais $OH^{\bullet}$ (Gutteridge, 1987).

O mecanismo pelo qual a oxima butano-2,3-dionatiosemicarbazona interfere na formação dos radicais $OH^{\bullet}$ está possivelmente relacionada à sua capacidade de neutralizar a

espécie reativa H_2O_2 (Figura 2, Manuscrito 2). Além disso, sugerimos que uma interação significativa entre íons Fe^{2+} e a oxima possa estar relacionada à sua capacidade de interferir na formação de radicais $OH^{\bullet}$. Esta hipótese é corroborada pela análise dos efeitos da oxima na reação entre a σ-fenantrolina e íons Fe^{+2} (Tabela 2, Manuscrito 2). Estes resultados sugerem que uma significativa interação ocorre entre íons Fe^{+2} e as moléculas da oxima diminuindo assim a reação dos íons Fe^{+2} com a σ-fenantrolina. A possível interação entre íons Fe^{+2} e as moléculas da oxima pode ser justificada pela presença de um átomo de enxofre em sua estrutura (Figura 1, Manuscrito 2). Compostos químicos contendo átomos de enxofre em sua estrutura podem estabelecer uma ligação com íons Fe^{2+} e atuarem com quelantes diminuindo a disponibilidade destes íons para reagir com outras biomoléculas (Miller e cols., 1990). A capacidade da oxima em atuar como um possível quelante de íons Fe^{2+} é um resultado bastante importante de nosso estudo, uma vez que compostos capazes de bloquear as reações oxidativas de componentes biológicos induzidas pelo ferro, como metal de transição, podem ser empregados como potenciais agentes antioxidantes (Miller e cols., 1990; Ryan e cols., 1993; Welch e cols., 2002).

O estudo dos efeitos tóxicos da oxima butano-2,3-dionatiosemicarbazona, a partir de experimentos realizados *in vivo* e *ex vivo*, sugerem que a oxima apresentou uma toxicidade pequena em camundongos. Esta conclusão baseia-se no fato de que o tratamento com a oxima não determinou alterações significativas nos níveis de tióis não protéicos (GSH e GSSG) (dado não apresentado) e na atividade da enzima δ-ALA-D (dado não apresentado) em preparações dos diferentes tecidos de camundongos. Desta forma, os resultados obtidos indicam que a oxima apresentou uma baixa toxicidade uma vez que os níveis de GSH/GSSG e a atividade da enzima δ-ALA-D, utilizados como parâmetros do dano oxidativo induzido pelo tratamento dos camundongos com a oxima, não foram significativamente alterados nas análises realizadas *ex vivo*. Além disso, a dose letal da oxima para cinqüenta por cento da amostra (LD_{50}) foi de 121 mg/kg (Figura 7, Manuscrito 2), um valor similar ao encontrado para outras oximas comumente utilizadas na clínica (Arena, 1979).

Os resultados obtidos nas análises realizadas *ex vivo* denotam a manutenção da atividade antioxidante da oxima butano-2,3-dionatiosemicarbazona em diminuir os níveis de TBARS basais e os induzidos por NPS em preparações de fígado e rim de camundongos (Tabela 3, Manuscrito 2). O emprego do NPS como agente pro-oxidante nas análises *ex vivo* foi baseada no fato de que este, ao reagir com agentes redutores, é degradado e libera radicais NO (Bates e cols., 1991). Os radicais NO podem então reagir com molécula de O_2^- formando

peroxinitritos ($ONOO^-$), os quais são moléculas altamente oxidantes e estão envolvidas no aumento do dano oxidativo tecidual (Villa e cols., 1994). É importante salientar que, além dos radicais NO, a degradação das moléculas de NPS também pode acarretar a liberação de complexos de pentacianoferratos, os quais também podem estar envolvidos na origem do dano oxidativo tecidual induzido por NPS (Rao e cols., 1991; Roncaroli e cols., 2005; Bates e cols., 1991). Apesar de a oxima apresentar uma significativa capacidade de interação com Fe^{2+}, como demonstrado nas análises *in vitro*, esta não foi capaz de reduzir significativamente os níveis de TBARS induzidos por Fe^{2+} nas análises *ex vivo*. A ausência de efeitos da oxima em reduzir os níveis de TBARS induzidos por Fe^{2+} pode ser explicada por uma possível mudança na capacidade de interação entre as moléculas da oxima e íons Fe^{2+} determinadas pela metabolização da oxima. Desta forma, com base no conjunto de resultados obtidos, acreditamos que o principal mecanismo envolvido na atividade antioxidante da oxima em reduzir significativamente os níveis de TBARS induzidos por NPS é a sua capacidade de neutralizar de maneira significativa a formação dos radicais NO (Figura 3, Manuscrito 2).

Por fim, a incapacidade da oxima butano-2,3-dionatiosemicarbazona em reduzir os níveis de TBARS, basais ou induzidos por NPS ou Fe^{2+}, em preparações de cérebro de camundongos pode ser atribuída a sua reduzida capacidade de ultrapassar a barreira cérebro-sangue. A reduzida capacidade de ultrapassar esta barreira é uma característica comum entre as oximas, e estudos conduzidos com o objetivo de mensurar esta característica revelam que a pralidoxima, uma oxima frequentemente utilizada na clínica, apresenta uma capacidade de ultrapassar a barreira cérebro-sangue de apenas aproximadamente 10% de suas moléculas (Sakurada e cols., 2003)

5. CONCLUSÕES

De acordo com os resultados apresentados nesta dissertação podemos concluir que:

- A atividade antioxidante da oxima 3-(fenil hidrazona) butano-2-ona está possivelmente relacionada a formação de metabólitos ativos.
- A atividade antioxidante da oxima 3-(fenil hidrazona) butano-2-ona não está relacionada à sua capacidade de interferir na reação $Fe^{2+} + H_2O_2$, e a ausência de efeitos nesta reação possivelmente está relacionada à sua incapacidade de interação íons Fe^{2+}.
- A atividade antioxidante da oxima 3-(fenil hidrazona) butano-2-ona não pode ser diretamente atribuída a sua habilidade de neutralizar ERs como os radicais $OH^{\bullet}$ e NO, corroborando a hipótese da formação de metabólitos ativos.
- A análise dos efeitos tóxicos da oxima 3-(fenil hidrazona) butano-2-ona, a partir dos experimentos realizados *in vitro* e *ex* vivo sugerem que a oxima apresentou uma toxicidade pequena em camundongos, uma vez que a determinação dos níveis de NPSH e da atividade da enzima δ-ALA-D não foram significativamente alterados pela ação da oxima tanto nas análises realizadas *in vitro* (atividade da enzima δ-ALA-D apenas) quanto nas realizadas *ex vivo*.
- A meia-vida plasmática curta da oxima 3-(fenil hidrazona) butano-2-ona pode ser considerada uma explicação para a ausência de efeitos antioxidantes da oxima nos resultados das análises *ex vivo*.
- Um dos possíveis mecanismos envolvido na gênese das propriedades antioxidantes da oxima butano-2,3-dionatiosemicarbazona refere-se a sua capacidade de neutralizar diferentes formas de ERs como o H_2O_2, e o radical $OH^{\bullet}$, além de neutralizar a formação do radical NO.
- Além da capacidade de neutralizar diretamente os radicais $OH^{\bullet}$, a oxima butano-2,3-dionatiosemicarbazona também interfere significativamente na formação destes radicais.
- O mecanismo pelo qual a oxima butano-2,3-dionatiosemicarbazona interfere na formação dos radicais $OH^{\bullet}$ está possivelmente relacionada à sua capacidade de neutralizar a espécie reativa H_2O_2.

- Os resultados obtidos sugerem que uma significativa interação ocorre entre íons Fe^{+2} e as moléculas da oxima butano-2,3-dionatiosemicarbazona, a qual pode estar relacionada à sua capacidade de interferir na formação de radicais $OH^{\bullet}$.
- A possível interação entre íons Fe^{+2} e as moléculas da oxima butano-2,3-dionatiosemicarbazona pode ser justificada pela presença de um átomo de enxofre em sua estrutura.
- A análise dos efeitos tóxicos da oxima butano-2,3-dionatiosemicarbazona, a partir dos experimentos realizados *ex* vivo sugerem que a oxima apresentou uma toxicidade pequena em camundongos, uma vez que os níveis de GSH/GSSG e a atividade da enzima δ-ALA-D, utilizados como parâmetros do dano oxidativo induzido pelo tratamento dos camundongos com a oxima, não foram significativamente alterados nas análises realizadas *ex vivo*.
- Os resultados obtidos nas análises realizadas *ex vivo* denotam a manutenção da atividade antioxidante da oxima butano-2,3-dionatiosemicarbazona em diminuir os níveis de TBARS basais e os induzidos por NPS em preparações de fígado e rim de camundongos.
- O principal mecanismo envolvido na atividade antioxidante *ex vivo* da oxima butano-2,3-dionatiosemicarbazona em reduzir significativamente os níveis de TBARS induzidos por NPS é a sua capacidade de neutralizar de maneira significativa a formação dos radicais NO.
- A ausência de efeitos *ex vivo* da oxima butano-2,3-dionatiosemicarbazona em reduzir os níveis de TBARS induzidos por Fe^{2+} pode ser explicada por uma possível mudança na capacidade de interação entre as moléculas da oxima e íons Fe^{2+} determinadas pela metabolização da oxima.
- A incapacidade da oxima butano-2,3-dionatiosemicarbazona em reduzir os níveis de TBARS basais ou induzidos (NPS ou Fe^{2+}), em preparações de cérebro de camundongos, pode ser atribuída a sua reduzida capacidade de ultrapassar a barreira cérebro-sangue.

6. PERSPECTIVAS

Baseado nos resultados apresentados nessa dissertação faz-se necessário:

- Analisar os efeitos tóxicos e antioxidantes das oximas 3-(fenil hidrazona) butano-2-ona e butana-2,3-dionatiosemicarbazona quando administradas por diferentes vias de administração, como as vias intramuscular (i.m.), e intraperitoneal (i.p.).
- Investigar a capacidade das oximas estudadas em reativar as enzimas ChEs oriundas de diferentes tecidos de ratos, camundongos, e também de eritrócitos humanos inibidas por diferentes pesticidas OPs.
- Investigar os efeitos de diferentes pesticidas OPs no desenvolvimento de dano oxidativo em diferentes tecidos de ratos, camundongos, e também em eritrócitos humanos.
- Analisar os efeitos antioxidantes das oximas 3-(fenil hidrazona) butano-2-ona e butana-2,3-dionatiosemicarbazona em modelos de dano oxidativo induzido por pesticidas OPs.
- Investigar os efeitos de novas oximas sintetizadas em relação a sua capacidade de reativar as enzimas ChEs, e às suas propriedades tóxicas e antioxidantes.

7- REFERÊNCIAS BIBLIOGRÁFICAS

ABDOLLAHI, M.; RANJBAR, A.; SHADNIA, S.; NIKFAR, S.; REZAIE, A. Pesticides and oxidative stress: a review. **Med. Sci. Monit.**, 10(6):141-147; 2004.

ADAM, J.P. Imines, enamines and oximes. Comtemp. **Org. Synth.**, 4(6): 517-543; 1997.

ALBERTS, B.; JOHNSON, A.; LEWIS, J.; RAFF,M.; ROBERTS, K.; WALTER, P. **Molecular Biology of the cell.** 3rd ed., New York & London: Garland Publishing; 1994.

ARENA, J.M. **Poisoning, Toxicology, Symptoms, Treatments.** 4th ed., Springfield Ill, Charles C.Thomas, p. 133; 1979.

AUGUSTO, O.; BONINI, M.G.; AMANSO, A.M.; LINARES, E.; SANTOS, C.C.; MENEZES, S.L.D. Nitrogen dioxide and carbonate radical anion: two emerging radicals in biology. **Free Rad. Biol. Med.**, 32:841–859; 2002.

BANERJEE, B.D.; SETH, V.; BHATTACHARYA, A.; PASHA, S.T.; CHAKRABORTY, A.K. Biochemical effects of some pesticides on lipid peroxidation and free-radical scavengers. **Toxicol. Lett.**, 107:33–47; 1999.

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

BATES, J.N.; BAKER, M.T.; GUERRA, Jr.R.; HARRISON, D.G. Nitric oxide generation from nitroprusside by vascular tissue. Evidence that reduction of the nitroprusside anion and cyanide loss is required. **Biochem. Pharmacol.**, 42:S157-S165; 1991.

BRAUGHLER, J.M.; DUNCAN, L.A.; CHASE, R.L. The involvement of iron in lipid peroxidation. Importance of ferric to ferrous ratios in initiation. **J. Biol. Chem.**, 261:10282-10289; 1986.

CHOI, C.W.; KIM, S.C.; HWANG, S.S.; CHOI, B.K.; AHN, H.J.; LEE, M.Y.; PARK, S.H; KIM, S.K. Antioxidant activity and free radical scavenging capacity between Korean

medicinal plants and flavonoids by assay-guided comparison. **Plant Sci.**, 153:1161-1168; 2002.

DEDEOGLU, A.; FERRANTE, R.J.; ANDREASSEN, O.A.; DILLMANN, W.H.; BEAL, M.F. Mice overexpressing 70-kda heat shock protein show increased resistance to malonate and 3-nitropropionic acid. **Exp. Neurol.**, 176:262– 265; 2002.

DOWLA, H.A.; PANEMANGALORE, M.; BYERS, M.E. Comparative inhibition of enzymes of human erythrocytes and plasma *in vitro* by agricultural chemicals. **Arch. Environ. Contam. Toxicol.**, 31:107–114; 1996.

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

EDDLESTON, M.; MOHAMED, F.; DAVIES, J.O.J.; EYER, P.; WOREK, F.; SHERIFF, M.H.R.; BUCKLEY, N.A. Respiratory failure in acute organophosphorus pesticide self-poisoning. **Q. J. Med.**, 99:513–22; 2006.

EMERY, T. **Iron and your health**: facts and fallacies. Boca Raton, FL: CRC Press; 1991.

FARINA, M.; FOLMER, V.; BOLZAN, R.; ANDRADE, L.H.; ZENI, G. Selenoxides inhibit d-aminolevulinic acid dehydratase. **Toxicol. Lett.**, 119:27-37; 2001.

FOLMER, V.; SOARES, J.C.M.; GABRIEL, D.; ROCHA, J.B.T. A high fat diet inhibits δ-aminolevulinate dehydratase and increases lipid peroxidation in mice (Mus musculus). **J. Nutr.**, 133(7):2165-2170; 2003.

FORTUNATO, J.J.; FEIER, G.; VITALI, A.M.; PETRONILHO, F.C.; DAL-PIZZOL, F.; QUEVEDO, J. Malathion induced oxidative stress in rat brain regions. **Neurochem. Res.**, 31:671-678; 2006.

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

GREENE, J.G.; PORTER, R.H.P.; ELLER, R.V.; GREENAMYRE, J.T. Inhibition of succinate dehydrogenase by malonic acid produces an excitotoxic lesion in rat striatum. **J. Neurochem.**, 61:1151-1154; 1993.

GREENWOOD, N.N.; EARNSHAW, A. **Chemistry of the Elements**, 2nd ed., Butterworth-Heinemann, Oxford, UK. A great description of properties & chemistry of H_2O_2; 1997.

GULTEKIN, F.; OZTURK, M.; AKDOGAN, M. The effect of organophosphate insecticide chlorpyrifos-ethyl on lipid peroxidation and antioxidant enzymes (*in vitro*). **Arch. Tox.**, 74: 533-568; 2000.

GUPTA, R.C.; MILATOVIC, D.; DETTBARN, W.D. Depletion of energy metabolites following acetylcholinesterase inhibitor-induced sattus epilepticus: Protection by antioxidants. **Neurotoxicol.**, 22: 271-282; 2001.

GUTTERIDGE, J.M.C. Ferrous-salt-promoted damage to deoxyribose and benzoate. The increased effectiveness of hydroxyl-radical scavengers in the presence of EDTA. **Biochem. J.**, 243(3):709–714; 1987.

GUTTERIDGE, J.M.C.; HALLIWELL, B. Antioxidants in nutrition, Health and Disease. **Annu. Rev. Nutr.**, 16:33-50; 1994.

HALLIWELL, B. Oxidative stress and neurodegeneration: Were are we now? **J. Neuroch.**, 97:1634–1658; 2006.

HALLIWELL, B.; GUTTERIDGE, J. M. C. **Free radicals in Biology and Medicine**. 2ª ed, New York: Clerendon Press, 1989.

HERNÁNDEZ, A.F.; LÓPEZ, O.; RODRIGO, L.; GIL, F.; PENA, G.; SERRANO, J.L.; PARRÓN, T.; ÁLVAREZ, J.C.; LORENTE, J.A.; PLA, A. Changes in erythrocyte enzymes in humans long-term exposed to pesticides. Influence of several markers of individual susceptibility. **Toxicol. Lett.**, 159:13-21; 2005.

HOWLAND, M.A., AARON, C. **Pralidoxime**. In: Goldfrank, L., Flomenbaum, N., Weisman, R., Howland, M.A., Hofman, R. (Eds.), Emergencies Toxicology. Applenton & Lange, USA. 1445-1449; 1999.

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

LÓPEZ, O.; HERNÁNDEZ, A.F.; RODRIGO, L.; GIL, F.; PENA, G.; SERRANO, J.L.; PARRÓN, T.; VILLANUEVA, E.; PLA, A. Changes in antioxidant enzymes in humans with long-term exposure to pesticides. **Toxicol. Lett.**, 171:146-153; 2007.

MACIEL, E.N.; BOLZAN, R.C.; BRAGA, A.L.; ROCHA, J.B.T. Diphenyl diselenide and diphenyl ditelluride differentialy effect d-aminolevulinate dehydratase from liver, kidney and brain of mice. **J. Biochem. Toxicol.**, 14:310-319; 2000.

MARAGOS, W.F.; SILVERSTAIN, F.S. The mitochondrial inhibitor malonate enhances NMDA toxicity in the neonatal rat striatum. **Develop. Brain Res.**, 88:117-121; 1995.

MILLER, D.M.; BUETTNER, G.R.; AUST, S.D. Transition metals as catalysts of "autoxidation" reactions. **Free Rad. Biol. Med.**, 8:95–108; 1990.

MINOTTI, G.; AUST, S.D. An investigation into the mechanism of citrate-Fe^{2+}-dependent lipid peroxidation. **Free Rad. Biol. Med.**, 3:379-387; 1987.

MINOTTI, G., AUST, S.D. Redox cycling of iron and lipid peroxidation. **Lipids**, 27:219-226; 1992.

NIGG, H.N.; KNAAK, J.B. Blood cholinesterases as human biomarkers of organophosphorus pesticide exposure. **Rev. Environm. Contam. Toxicol.**, 163:29-111; 2000.

NORIEGA, G.O.; GONZALES, S.; TOMARO, M.L.; BATLE, A.M. Paraquat-generated oxidative stress in rat liver induces hemeoxygenase- 1 and aminolevulinic acid synthase. **Free Radic. Res.**, 36:633–639; 2002.

OHKAWA, H.; OHISHI, N.; YAGI, K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. **Anal. Biochem.**, 95:351- 358; 1979.

OUBIDAR, M.; BOQUILLON, M.; CHRISTINE M.; CHRISTINE B.; BELEY, A.; BRALET, J. Effect of intracellular iron loading on lipid peroxidation of brain slices. **Free Radic. Biol. Med.**, 21:763-769; 1996.

PACHER, P.; BECKMAN, J.S.; LIAUDET, L. Nitric oxide and peroxynitrite in health and disease. **Physiol. Rev.**, 87(1):315–424; 2007.

PANEMANGALORE, M.; DOWLA, H.A.; BYERS, M.E. Occupational exposure to agricultural chemicals: effect on the activities of some enzymes in the blood of farm workers. **Int. Arch. Occup. Environ. Health**, 72:84–88; 1999.

PEROTTONI, J.; MEOTTI F.C.; FOLMER, V.; PIVETTA, L.; NOGUEIRA, C. W.; ZENI, G.; ROCHA, J.B.T. Ebselen and diphenyl diselenide do not change the inhibitory effect of

lead acetate on delta-aminolevulinate dehidratase. **Environ. Toxicol. Pharmacol.**, 19:239–248; 2005.

PUNTEL, R.L.; ROOS, D.H.; PAIXÃO, M.W.; BRAGA, A.L., ZENI, G.; NOGUEIRA, C.W.; ROCHA, J.B.T. Oxalate modulates thiobarbituric acid reactive species (TBARS) production in supernatants of homogenates from rat brain, liver and kidney: Effect of diphenyl diselenide and diphenyl ditelluride. **Chem. Biol. Interact.**, 165:87-98; 2007.

RAO, D.N.; ELGUINDI, S.; O'BRIEN, P.J. Reductive metabolism of nitroprusside in rat hepatocytes and human erythrocytes. **Arch. Biochem. Biophys.**, 286:30–37; 1991.

RONCAROLI, F.; ELDIK, R.V.; OLABE, J.A. Release of NO from reduced nitroprusside ion. Iron-dinitrosyl formation and NO-disproportionation reactions. **Inorg. Chem.**, 44:2781–2790; 2005.

RYAN, T.P.; MILLER, D.M.; AUST, S.D. The role of metals in the enzymatic and nonenzymatic oxidation of epinephrine. **J. Biochem. Toxicol.**, 8:33–39; 1993.

SAKURADA, K.; MATSUBARA, K.; SHIMIZU, K.; SHIONO, H.; SETO, Y.; TSUGE, K.; YOSHINO, M.; SAKAI, I.; MUKOYAMA, H.; TAKATORI, T. Pralidoxime iodide (2-PAM) penetrates across the blood–brain barrier. **Neurochem. Res.**, 28:1401–1407; 2003.

SCHULZ, J.B.; HUANG, P.L.; MATTHEWS, R.T.; PASSOV, D.; FISHMAN, M.C.; BEAL, M.F. Striatal malonate lesions are attenuated in neuronal nitric oxide synthase knockout mice. **J. Neurochem.**, 67:430-433; 1996.

SHEEHAN, D.; MEADE, G.; FOLEY, V.; DOWD, C. Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. **Biochem. J.**, 360(Pt 1):1–16; 2001.

SIES, H. Oxidative stress: oxidants and antioxidants. **Exp. Physiol.**, 82(2):291-5; 1997.

SILVA, A.P.; FARINA, M.; FRANCO, J.L.; DAFRE, A.L.; KASSA, J.; KUCA, C. Temporal effects of newly developed oximes (K027, K048) on malathion-induced acetylcholinesterase inhibition and lipid peroxidation in mouse prefrontal cortex. **Neurotoxicol.**, 29:184-189; 2008.

SILVA, A.P.; MEOTTI, F.C; SANTOS, A.R.S.; FARINA, M. Lactational exposure to malathion inhibits brain acetylcholinesterase in mice. **Neurotoxicol.**, 27:1101-1105; 2006.

STENZEL, J.; WOREK, F.; EYER, P. Preparation and characterization of dialkylphosphoryl-obidoxime conjugates, potent anticholinesterase derivatives that are quickly hydrolyzed by human paraoxonase (PON1192Q). **Biochem. Pharmacol.**, 74:1390-1400; 2007.

TIMBRELL, J. **Principles of Biochemical Toxicology**. 3ª ed, London: Taylor & Francis, 2000.

VERMA, R.S. Chlorpyrifos-induced alterations in levels of thiobarbituric acid reactive substances and glutathione in rat brain. **Ind. J. Exp. Biol.**, 39:174–77; 2001.

VERTUANI, S.; ANGUSTI, A.; MANFREDINI, S. The antioxidants and pro-antioxidants network: an overview. **Curr. Pharm. Des.**, 10(14):1677–94; 2004.

VILLA, L.M.; SALAS, E.; DARLEY-USMAR, V.M.; RADOMSKI, M.W.; MONCADA, S. Peroxynitrite induces both vasodilatation and impaired vascular relaxation in the isolated perfused rat heart. **Proc. Natl. Acad. Sci. US A.**, 91:12383-12387; 1994.

WELCH, K.D.; DAVIS, T.Z.; VAN EDEN, M.E.; AUST, S.D. Deleterious iron-mediated oxidation of biomolecules. **Free Rad. Biol. Med.**, 32(7):577-583; 2002.

WIENER, S.W.; HOFFMAN, R.S. Nerve agents: a comprehensive review. J**. Intensive Care Med.**, 19:22–37; 2004.

WOREK, F.; AURBEK, N.; KOLLER, M.; BECKER, C.; EYER, P.; THIERMANN, H. Kinetic analysis of reactivation and aging of human acetylcholinesterase inhibited by different phosphoramidates. **Biochem. Pharmacol.**, 73(11):1807-1817; 2007.

WOREK, F.; THIERMANN, H.; SZINICZ, L.; EYER, P. Kinetic analysis of interactions between human acetylcholineserase, structurally different organophosphorus compounds and oximes. **Biochem. Pharmacol.**, 68:2237–2248; 2004.