UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM FARMACOLOGIA

AÇÃO ANTIOXIDANTE E NEUROPROTETORA DE COMPOSTOS PIRAZOLÍNICOS INÉDITOS

DISSERTAÇÃO DE MESTRADO

Daniele Moreira Martins

Santa Maria, RS, Brasil

2008

AÇÃO ANTIOXIDANTE E NEUROPROTETORA DE DERIVADOS PIRAZOLÍNICOS INÉDITOS

por

Daniele Moreira Martins

Dissertação apresentada ao Programa de Pós-Graduação em Farmacologia, Área de Concentração em Farmacologia, Linha de Pesquisa em Neuropsicofarmacologia e Imunofarmacologia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de
Mestre em Farmacologia.

Orientador: Profa. Dra. Tatiana Emanuelli

Santa Maria, RS, Brasil
2008

Universidade Federal de Santa Maria
Centro de Ciências da Saúde
Programa de Pós-Graduação em Farmacologia

A Comissão Examinadora, abaixo assinada,
aprova a Dissertação de Mestrado

AÇÃO ANTIOXIDANTE E NEUROPROTETORA DE DERIVADOS PIRAZOLÍNICOS INÉDITOS

elaborada por
Daniele Moreira Martins

como requisito parcial para obtenção do grau de
Mestre em Farmacologia

COMISSÃO EXAMINADORA:

_____
Tatiana Emanuelli, Dra.
(Presidente/Orientador)

_____
Vanderlei Folmer, Dr. (UNIPAMPA)

_____
Juliano Ferreira, **Dr.** (UFSM)

Santa Maria, 28 de março de 2008.

AGRADECIMENTOS

Agradeço em primeiro lugar a Deus pela vida, pelas oportunidades que colocou no meu caminho, e por ter oportunizado que tantas pessoas especiais estejam sempre no meu caminho.

Aos meus pais, Antônio e Elva pelos valores, apoio, dedicação, confiança e por sempre terem disponibilizado condições tanto morais quanto materiais para que eu sempre pudesse seguir em frente na busca dos meus objetivos.

Aos meus irmãos, Alexandre e Luciano pelo incentivo, compreensão e amizade nas horas mais difíceis.

À minha orientadora Prof[a] Tatiana Emanuelli por ter me aceito em seu grupo de pesquisa, pelo esforço dedicado, pela confiança, pela paciência, pela amizade e por todos os ensinamentos transmitidos. A você o meu agradecimento e profunda admiração pela pessoa que és, um exemplo de profissional dedicado à pesquisa.

À minha amiga Tatiana Noal pela amizade, carinho e compreensão, sempre presente nas inúmeras horas em que mais precisei. Obrigada de coração!!!

Aos meus amigos Marília, Clarissa, Viviane Ilha, Ana Rita, Ana Paula Daniel, Ana Cristina, Rosselei, Osmar, Rafael Vivian e Prof[a] Ionara Pizzutti que mesmo um pouco distantes não deixaram de estar presentes, sendo fundamentais na minha vida.

Às minhas estagiárias, Bruna Torres, Juliana, Patrícia, Sabrina e Nardeli pela contribuição fudamental para que este trabalho fosse executado e à Cristiane

Denardin pela colaboração fundamental na segunda etapa do trabalho. O meu agradecimento pelo empenho de cada uma, bem como pela amizade e pelo auxílio durante as intermináveis horas de trabalho no laboratório. Muito Obrigada!!

Às minhas colegas Angélica e Luciana pela amizade, coleguismo e companheirismo durante o período do curso.

Aos colegas e amigos do Nidal, inclusive aqueles que já seguiram seus caminhos, pelo carinho, amizade e conhecimentos compartilhados e pelos momentos de descontração durante o convívio no laboratório, em especial a Jucieli, Roberta Barbosa, Cristiane Denardin, Vivian, Greicy, Paula e Cristiane Portes.

Aos professores da Pós-Graduação em Farmacologia pelos ensinamentos passados e pela contribuição na minha formação.

À Prof [a] Maribel Antonello Rubin pelos conhecimentos a mim passados sobre as análises de comportamento, pela ajuda nos ensaios e principalmente por ceder o seu Laboratório para a execução das análises.

Aos professores Juliano Ferrreira, Vanderlei Folmer e Marilise Escobar Bürguer por aceitarem o convite para compor a banca examinadora desta dissertação.

Aos funcionários do Biotério Central pelo fornecimento dos animais e demais auxílios prestados.

Ao NUQUIME, responsável pela síntese das drogas, especialmente ao Pablo, pela sua dedicação ao trabalho de síntese.

A UFSM e ao Programa de Pós-graduação em Farmacologia pela oportunidade de realizar este curso.

A CAPES, pela bolsa de estudos concedida.

A todos aqueles que de alguma forma contribuíram para a realização desse trabalho.

RESUMO

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

Ação Antioxidante e Neuroprotetora de Derivados Pirazolínicos Inéditos

Autora: Daniele Moreira Martins
Orientadora: Tatiana Emanuelli
Data e Local da Defesa: Santa Maria, 28 de março de 2008

O estresse oxidativo está envolvido em diversas doenças neurodegenerativas importantes, tais como a doença de Alzheimer, a doença de Parkinson e a esclerose lateral amiotrófica. O estresse oxidativo parece estar envolvido na patologia da demência/amnésia, tendo sido sugerido que as alterações cerebrais decorrentes deste causam danos ao sistema colinérgico muscarínico e que desta forma desencadeiam a doença de Alzheimer. A escopolamina, um antagonista muscarínico, tem sido usado para induzir amnésia em animais, em um modelo experimental para a triagem de drogas que poderiam ser úteis no tratamento da demência. O principal objetivo deste estudo foi avaliar o possível efeito antioxidante *in vitro* de uma série de derivados pirazolínicos recém sintetizados: (**1**) 5-hidroxi-3-metil-5-trifluorometil-4,5-diidro-1*H*-carbaldeido-pirazol, (**2**) 5-hidroxi-3-metil-5-trifluorometil-4,5-diidro-1*H*-1-acetil-pirazol, (**3**) 5-hidroxi-3-metil-5-trifluorometil-4,5-diidro-1*H*-carboxiamida-pirazol, (**4**) 5-hidroxi-3-metil-5-trifluorometil-4,5-diidro-1*H*-1-benzoil-pirazol, (**5**) 5-hidroxi-3-metil-5-trifluorometil-4,5-diidro-1*H*-1-(2-hidroxibenzoil)-pirazol e (**6**) 5-hidroxi-3-metil-5-trifluorometil-4,5-diidro-1*H*-1-(4-methoxibenzoil)-pirazol. Além disso, considerando o possível envolvimento do estresse oxidativo na demência, foi avaliada a capacidade do composto mais efetivo *in vitro*, em prevenir o déficit de memória e o estresse oxidativo em um modelo de amnésia induzida por escopolamina. O derivado pirazolínico (**5**) apresentou maior capacidade antioxidante *in vitro*, pois foi o mais efetivo para reduzir a lipoperoxidação (TBARS) basal e induzida pelos pró-oxidantes ferro, peróxido de hidrogênio e nitroprussiato de sódio, tendo efeitos significativos a partir de 15 µM ($p<0,05$). O composto (**5**) também protegeu a glutationa da oxidação induzida por peróxido de hidrogênio, tendo efeito significativo na concentração de 150 µM ($p<0,05$). Este composto também foi o que teve maior atividade antioxidante total, demonstrada pela sua capacidade de remover o radical 1,1-difenil-2-picrilhidrazil (DPPH). Os compostos (**1**) e (**4**) também reduziram a lipoperoxidação basal e induzida por ferro e nitroprussiato de sódio, tendo efeitos significativos a partir de 15 µM ($p<0,05$). O composto (**2**) apresentou a maior capacidade de redução de ferro ($p<0,05$). A administração de escopolamina 30 min antes do treino provocou amnésia, medida como a redução na latência para descer da plataforma no teste de esquiva inibitória ($p<0.05$). O pré-tratamento com o composto (**5**) 30 min antes da escopolamina não apresentou efeito *per se* na latência, mas preveniu o efeito amnésico da escopolamina, na dose de 100 µmol/kg ($p<0.05$). Não foi observado efeito significativo da escopolamina ou do composto (**5**) em qualquer dos marcadores de estresse oxidativo avaliados (substâncias reativas ao ácido tiobarbitúrico, grupos tiólicos não protéicos e atividade das enzimas superóxido dismutase e catalase), sugerindo que o efeito protetor do composto (**5**) não está relacionado à sua atividade antioxidante. Os resultados obtidos demonstram que o composto (5) apresenta atividade antioxidante *in vitro* e neuroprotetora em um modelo de amnésia, sugerindo que este composto pode ser promissor para o tratamento da doença de Alzheimer. No entanto, outros estudos são necessários para

elucidar os mecanismos envolvidos na ação anti-amnésica deste composto, bem como o seu efeito em outros modelos de demência.

Palavras-chave: pirazóis; SNC; estresse oxidativo; superóxido dismutase; catalase; escopolamina; peroxidação lipídica

ABSTRACT

Master Dissertation
Graduate Course on Pharmacolgy
Federal University of Santa Maria, RS, Brazil

Antioxidant and Neuroprotective Activity of New Pyrazoline Derivatives

Author: Daniele Moreira Martins
Adviser: Tatiana Emanuelli
Date and Place of the Defense: Santa Maria, March 28, 2008

Oxidative stress is involved in several neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis. Oxidative stress seems to be involved in the pathology of dementia/amnesia. It has been suggested that oxidative stress impairs the muscarinic cholinergic system triggering Alzheimer's disease. The muscarinic antagonist scopolamine has been used to induce amnesia in animals. This experimental model has been used in screening anti-amnesic drugs that could be useful for the treatment of dementia. The aim of this study was to evaluate the possible *in vitro* antioxidant effect of a series of pyrazoline derivatives newly synthesized: (**1**) 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-carbaldehyde-pyrazole, (**2**) 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-1-acetyl-pyrazole, (**3**) 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-carboxyamide-pyrazole, (**4**) 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-1-benzoyl-pyrazole, (**5**) 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-1-(2-hydroxybenzoyl)-pyrazole and (**6**) 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-1-(4-methoxybenzoyl)-pyrazole. Besides, considering the possible involvement of oxidative stress in dementia, the compound that was the most effective *in vitro* was assessed concerning to its ability to prevent the memory deficit and oxidative stress in a scopolamine-induced amnesia model. Compound (**5**) had the highest antioxidant capacity *in vitro*, since it reduced lipid peroxidation (TBARS) basal and stimulated by the pro-oxidants iron, hydrogen peroxide and sodium nitroprusside, having significant effects from 15 µM onwards ($p<0.05$). Compound (**5**) also protected against hydrogen peroxide-induced glutathione oxidation, with a significant effect at the concentration of 150 µM ($p<0.05$). This compound also had the highest total antioxidant activity, demonstrated by its ability to remove the radical 1,1-dyphenyl-2-pycrylhydrazyl (DPPH). Compounds (**1**) and (**4**) also reduced lipid peroxidation basal and stimulated by iron and sodium nitroprusside, having significant effects from 15 µM onwards ($p<0.05$). Compound (**2**) had the highest ability to reduce iron ($p<0.05$). Scopolamine administration 30 min before training session resulted in shorter latency to step-down during the test session of the inhibitory avoidance task ($p<0.05$). Pretreatment with pyrazole compound (**5**) had no effect *per se* on the step-down latency. However, pretreatment with compound (**5**) (100 µmol/kg) 30 min before scopolamine did prevent the amnesic effect of scopolamine ($p<0.05$). No significant effect of scopolamine or pyrazole treatment was observed on any of the oxidative stress markers evaluated (thiobarbituric acid reactive substances, non-protein sulfhydrylic groups content and activity of enzymes superoxide dismutase and catalase) suggesting that the protective effect of compound (**5**) was not related to a possible antioxidant activity. Results revealed that pyrazole compound (**5**) has *in vitro* antioxidant activity as well as neuroprotective activity in a model of amnesia. These findings suggest that compound (**5**) could be a promising drug for the treatment of Alzheimer´s disease. However, further studies are needed to elucidate the mechanisms involved in the antiamnesic effect of this compound, as well as its effect on other dementia models.

Keywords: pyrazoles; CNS; oxidative stress; superoxide dismutase; catalase; scopolamine; lipid peroxidation

LISTA DE FIGURAS

LISTA DE ANEXOS

LISTA DE ABREVIATURAS

ANOVA – Análise de variância

IC_{50} – Concentração Inibitória 50

DNA – Ácido desoxirribonucléico

DNA_n – Ácido desoxirribonucléico nuclear

DNA_{mt} - Ácido desoxirribonucléico mitocondrial

NO – Óxido nítrico

EROs – Espécies reativas de oxigênio

GPx – Glutationa peroxidase

GSH – Glutationa

ip – Intraperitoneal

MDA – Malondialdeído

Prxs – Peroxirredoxinas

RNAm – Ácido ribonucléico mensageiro

SH – Grupos sulfidrílicos

SHNP – Grupos tiólicos não-protéicos

SOD – Superóxido dismutase

CAT – Catalase

TBARS – Substâncias reativas ao ácido tiobarbitúrico

SNP – nitroprussiato de sódio

SUMÁRIO

APRESENTAÇÃO

Os resultados que fazem parte desta dissertação são apresentados sob a forma de manuscritos, os quais se encontram no item RESULTADOS. As seções Material e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios manuscritos e representam na íntegra este estudo.

Os itens DISCUSSÃO E CONCLUSÃO, dispostos após os manuscritos, contêm interpretações e comentários gerais referentes ao presente estudo e relacionados aos manuscritos deste trabalho.

As REFERÊNCIAS BIBLIOGRÁFICAS são relacionadas às citações que aparecem nos itens INTRODUÇÃO, REVISÃO BIBLIOGRÁFICA e DISCUSSÃO desta dissertação.

1 INTRODUÇÃO

Espécies reativas incluem moléculas com um elétron desemparelhado em sua órbita externa (radicais livres), bem como moléculas sem elétrons desemparelhados, mas que também possuem grande reatividade com moléculas orgânicas. As espécies reativas são produzidas no curso de diversas reações fisiológicas, tais como na respiração celular, nos macrófagos durante a fagocitose, entre outras. No entanto, um desequilíbrio entre a formação de espécies reativas e a atividade dos sistemas de defesa antioxidante endógenos, pode levar a danos oxidativos em biomoléculas tais como, lipídios, proteínas e DNA. Essa situação é denominada estresse oxidativo e pode prejudicar o funcionamento de organelas, tais como a mitocôndria, e danificar a membrana celular, levando à morte celular (Halliwell & Gutteridge, 1999).

Sabe-se que o estresse oxidativo está envolvido em diversas doenças importantes, especialmente no sistema nervoso, tais como a doença de Alzheimer (Butterfield *et al*., 2001), a doença de Parkinson (Fahn & Cohen, 1992), a esclerose lateral amiotrófica (Coyle & Putfarcken, 1993), a demência (Cruz *et al*., 2003), entre outras. Alguns estudos apontam o envolvimento do estresse oxidativo na patologia da demência/amnésia, sugerindo que as alterações cerebrais decorrentes deste causam danos ao sistema colinérgico muscarínico (Mattson & Pedersen, 1998) e que desta forma desencadeiam a doença de Alzheimer (Varadarajan *et al*., 2000; Castegna *et al*., 2003; Sultana *et al*., 2006b). Além disso, o estresse oxidativo também tem sido associado à disfunção cognitiva associada ao dano no aprendizado e memória, e tem sido proposto que o déficit de memória é acompanhado de mudanças nos índices/atividades dos marcadores de estresse oxidativo (El-Sherbiny *et al*., 2003).

Na doença de Alzheimer, alguns marcadores da peroxidação lipídica (malondialdeído, 4-hidroxinonenal, F2-isoprostanos), da oxidação protêica (proteína carbonil, nitrotirosina) e da oxidação do DNA (8-hidroxi-2-deoxiguanosina) estão presentes no tecido cerebral e tornam-se biomarcadores do dano oxidativo nesta doença (Praticò, 2005).

Há evidências de que compostos que atuam removendo radicais livres ou evitando a sua formação tem sido capazes de prevenir ou retardar o dano oxidativo

neuronal *in vitro* (Rosler *et al.*, 1998). Também existem alguns dados clínicos indicando a ação neuroprotetora de drogas que possuem atividade antioxidante, tais como gingko biloba, selegilina e vitamina E (Rosler *et al.*, 1998). Também alguns estudos sugerem que compostos podem reverter o dano oxidativo existente em doenças como demência/amnésia (El-Sherbiny *et al*., 2003).

Os compostos heterocíclicos pirazolínicos são drogas de origem sintética que se caracterizam por apresentar em sua estrutura um anel pirazolínico, o qual é um heterociclo de cinco membros, sendo que nas posições 1 e 2 têm-se átomos de nitrogênio. Vários compostos dessa classe apresentam atividades farmacológicas variadas, como ação analgésica, antipirética e antiinflamatória (Borne, 1995).

Alguns desses compostos podem apresentar atividade antioxidante por mecanismos indiretos (inibição de sistemas enzimáticos que produzem radicais livres) e, em alguns casos, por mecanismos diretos. No caso do mecanismo direto, foi demonstrado que compostos como 3,5-diaril-pirazolinas e pirazóis são capazes de inibir a oxidação de LDL *in vitro*, sendo que um destes compostos apresentou uma potência seis vezes maior que o probucol, um antioxidante sintético (Jeong *et al*., 2004). Diversos compostos pirazolínicos são inibidores da ciclooxigenase-2, enzima da via de biossíntese das prostaglandinas. A ativação desta via está envolvida em diversos processos fisiológicos, incluindo o processo inflamatório, e pode resultar em um aumento na produção de espécies reativas, causando danos oxidativos (Tsai *et al*., 1998). A inibição desta enzima resulta em atividade antiinflamatória, como já foi demonstrado para alguns compostos pirazolínicos em modelos animais (Maggio *et al*., 2001; Ranatunge *et al*., 2004).

Estudos demonstraram que os derivados pirazolínicos possuem ainda potencial antiinflamatório (de Souza *et al*., 2001), antipirético (Souza *et al*., 2002; Tomazetti *et al*., 2004) e atividade antinociceptiva (Mello *et al*., 1996; Frussa-Filho *et al*., 1996; de Souza *et al*., 2001; Tabarelli *et al*., 2003; Tabarelli *et al*., 2004; Prokopp, 2004., Godoy *et al*., 2004).

O presente estudo teve como objetivo avaliar o possível efeito antioxidante *in vitro* de uma série de derivados pirazolínicos recém sintetizados (figura 1). Além disso, considerando o possível envolvimento do estresse oxidativo na demência, foi avaliada a capacidade do composto mais efetivo *in vitro*, em prevenir o déficit de memória e o estresse oxidativo em um modelo de amnésia induzida por escopolamina.

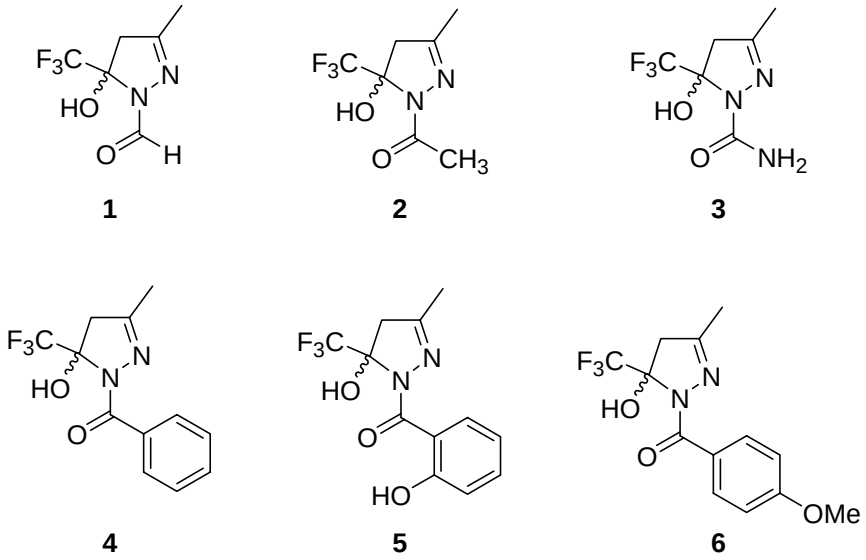


Figura 1: Estrutura química dos derivados pirazolínicos avaliados. **1)** 5-hidroxi-3-metil-5-trifluorometil-4,5-diidro-1*H*-carbaldeido-pirazol, (**2**) 5-hidroxi-3-metil-5-trifluorometil-4,5-diidro-1*H*-1-acetil-pirazol, (**3**) 5-hidroxi-3-metil-5-trifluorometil-4,5-diidro-1*H*-carboxiamida-pirazol, (**4**) 5-hidroxi-3-metil-5-trifluorometil-4,5-diidro-1*H*-1-benzoil-pirazol, (**5**) 5-hidroxi-3-metil-5-trifluorometil-4,5-diidro-1*H*-1-(2-hidroxibenzoil)-pirazol and (**6**) 5-hidroxi-3-metil-5-trifluorometil-4,5-diidro-1*H*-1-(4-metoxibenzoil)-pirazol

2 REVISÃO BIBLIOGRÁFICA

2.1 Espécies reativas e estresse oxidativo

As espécies reativas de oxigênio (ERO) são definidas como moléculas com um elétron desemparelhado em sua órbita externa (radicais livres), bem como moléculas sem elétrons desemparelhados, mas que também possuem grande reatividade com moléculas orgânicas. As EROs são geradas no curso de diversas reações fisiológicas, tais como na respiração celular, nos macrófagos durante a fagocitose, entre outras situações e exogenamente por agentes ambientais (Halliwell & Gutteridge, 1999). A formação de EROs conduzida a concentrações fisiológicas é necessária para a função celular normal, mas em quantidades excessivas pode levar ao estresse oxidativo (Nordberg & Arnér, 2001). O estresse oxidativo ocorre quando há um desequilíbrio entre fatores oxidantes e antioxidantes, a favor dos oxidantes, prejudicando a integridade celular (Sies, 2000). As EROs incluem um grande número de moléculas quimicamente reativas, como por exemplo o ânion radical superóxido ($O_2^{\cdot -}$), o peróxido de hidrogênio (H_2O_2) e o radical hidroxil ($^{\cdot}OH$). As moléculas anteriormente citadas e seu mecanismo de formação estão ilustrados na Figura 2.

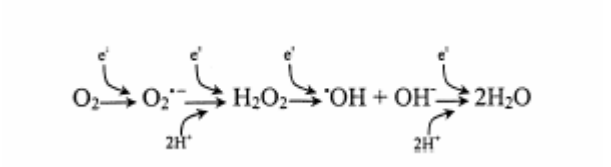


Figura 2 – Esquema de formação das espécies reativas de oxigênio, a partir do oxigênio molecular, com sucessivas transferências de elétrons (Nordberg & Arnér, 2001).

O radical hidroxil reage com componentes da molécula do DNA, danificando as bases púricas e pirimidínicas (Halliwell & Gutteridge, 1999). Também causa danos ao DNA e ao RNA, às proteínas dos lipídios e às membranas celulares do núcleo e mitocondrial (Halliwell & Gutteridge, 1999). No DNA, ele ataca tanto as bases nitrogenadas quanto a desoxirribose (Barreiros & David, 2006) e danifica também outras estruturas como ácidos graxos poliinsaturados, resíduos de fosfolipídeos, que são extremamente sensíveis a oxidação, desencadeando a peroxidação e oxidação de grupos tióis (Molavi & Mehta, 2004). A peroxidação

lipídica é uma forma proeminente e especialmente deletéria de lesão neuronal oxidativa, danificando membranas e gerando vários produtos secundários, tanto de cisão quanto de endociclização de ácidos graxos oxigenados que possuem atividade neurotóxica (Basset & Montine, 2003).

2.2 Estresse oxidativo e doenças neurodegenerativas

O estresse oxidativo tem sido associado a inúmeras doenças neurodegenerativas, como a doença de Alzheimer, Parkinson e esclerose lateral amiotrófica entre outras. No caso da doença de Alzheimer, estudos demonstram que muitos marcadores do dano oxidativo estão presentes em quantidades aumentadas nos tecidos cerebrais de pacientes. Um significante aumento de 8-desoxideoxiguanosina (8-OHdG) foi encontrado nos DNA mitocondrial e DNA nuclear isolados de áreas corticais e do cerebelo de pacientes com Alzheimer em comparação com os pacientes controle, particularmente em áreas do córtex parietal. Estes níveis se encontram mais aumentados no DNA mitocondrial do que no DNA nuclear, demonstrando que existe uma suceptibilidade da mitocôndria ao estresse oxidativo (Meccocci *et al*., 2005). Além disso, no cérebro de pacientes com Alzheimer encontram-se aumentados os níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS) nas áreas do córtex temporal e frontal quando comparado aos pacientes controle (Subbarao *et al*., 1990; Marcus *et al*., 1998). Somando-se a isso, alguns estudos também demonstram níveis aumentados de 4-hidroxinonenal (HNE) em múltiplas regiões cerebrais de pacientes com Alzheimer (Zarkovic, 2003), assim como níveis aumentados de grupos carbonila em áreas do hipocampo e do lóbulo parietal inferior.

A ocorrência do estresse oxidativo na doença de Alzheimer é suportada por estudos *post mortem* e também por estudos que demonstram a capacidade do estresse oxidativo em induzir a degeneração das células nigrais. Evidências sugerem que há um elevado índice de estresse oxidativo basal na substância negra parte compacta em cérebros normais, mas que há um aumento ainda maior destes índices na doença de Parkinson (Andersen, 2004). No entanto, outros fatores, envolvendo inflamação, mecanismos excitotóxicos e disfunção mitocondrial são importantes como desencadeadores da doença de Parkinson (Andersen, 2004). Na

doença de Parkinson, o estresse oxidativo contribui para a cascata de eventos que conduzem à degeneração dopaminérgica celular (Adam-Vizi, 2004).

2.3 Antioxidantes

É definido como antioxidante qualquer substância que quando presente em baixas concentrações, comparadas àquelas de um substrato oxidável, é capaz de retardar e/ou bloquear significativamente uma reação de oxidação. O termo substrato inclui proteínas, lipídeos e DNA. Também pode ser chamado de antioxidante o composto com capacidade de quebrar uma reação em cadeia da peroxidação de lipídeos (Agarwal *et al*., 2005).

Os antioxidantes são divididos em enzimáticos e não-enzimáticos. Os não-enzimáticos consistem de moléculas endógenas como a glutationa e NADPH e exógenas como o ácido ascórbico (Vitamina C), α-tocoferol (Vitamina E), ácido α-lipóico, carotenóides, flavonóides entre outros (Schreibelt *et al*., 2007). Dentre os antioxidantes não-enzimáticos destacam-se o ácido ascórbico e o α-tocoferol. O ácido ascórbico atua como removedor de espécies reativas como os radicais peroxil e hidroxil *in vitro*. É um potente removedor de oxigênio singlete, também atua na proteção de membranas e lipoproteínas contra a peroxidação lipídica induzida por espécies reativas presentes na fumaça do cigarro, por exemplo e inibe o dano oxidativo pela remoção de radicais gerados por certas drogas. Já o α-tocoferol atua como removedor de radicais peroxil, inibindo a reação de peroxidação lipídica (Halliwell & Gutteridge, 1999).Os enzimáticos incluem as enzimas endógenas superóxido dismutase (SODs) (McCord & Edeas, 2005), peroxiredoxinas (Prxs) (Dringen *et al*., 2005; Kim *et al*., 2007), glutationa peroxidase (GSHPx), tiorredoxina redutase (Conterato *et* al., 2007), catalase (CAT) (Valko *et al*., 2007), heme oxigenases (HOs) (Wagener *et al*., 2003; Ishii *et al*., 2000), NAD(P)H:quinona oxidoredutase 1 (NQO1) e NHR:quinona oxidoredutase 2 (NQO2) (Li & Jaiswal, 1992; Jaiswal, 2000; Iskander *et al*., 2006). As enzimas antioxidantes protegem as células aeróbicas e demais estruturas das injúrias oxidativas causadas por EROs, as quais são geradas durante o metabolismo normal (Fridovich, 1978) (Figura 3).

A primeira linha de defesa contra o estresse oxidativo é feita pelas enzimas superóxido dismutase (SODs), que é um grupo de metaloenzimas que catalisam a

dismutação do ânion superóxido ao oxigênio molecular e peróxido de hidrogênio (Johnson & Giulivi, 2005). As SODs existem em muitas formas, diferindo estruturalmente, pelo metal e pelo número de subunidades. Em humanos, existe expressão de três formas, a SOD citosólica cobre-zinco (SOD1/Cu/Zn) (McCord & Fridovich, 1969), a SOD mitocondrial manganês (SOD2/MnSOD) (McCord, 1976) e a SOD extracelular cobre-zinco (SOD3/CuZn) (Marklund, 1982). As SOD1 e SOD2 são bem expressas no sistema nervoso central (SNC), sendo que a última é principalmente encontrada em neurônios (Maier & Chan, 2002). A SOD3 é também expressa no SNC, mas em menores concentrações que a SOD1 e SOD2 (Marklund, 1984).

A catalase é uma enzima de defesa antioxidante intracelular que se localiza principalmente nos peroxissomas e em maior extensão no citosol das células dos mamíferos. A catalase catalisa a conversão de peróxido de hidrogênio a água e oxigênio molecular; tornando-se particularmente importante quando existe pouca disponibilidade de glutationa, tendo um papel fundamental no desenvolvimento da tolerância ao estresse oxidativo celular. No SNC, a expressão da catalase tem sido demonstrada para todos os tipos de células (Dringen *et al*., 2004).

A glutationa peroxidase constitui uma família de seleno-enzimas que detoxificam os peróxidos e o peróxido de hidrogênio pela oxidação de duas moléculas de glutationa. Dessa forma, seis tipos de GPx já foram identificadas nas células de mamíferos. A GPx1 é geralmente expressa no citosol e na matriz mitocondrial em praticamente todas as células, enquanto que a GPx2, GPx4, GPx5 e GPx6 são encontradas especificamente em órgãos e tecidos e a GPx3 é uma glicoproteína extracelular (Brigelius-Flohe, 1999; Herbette *et al*., 2007). No cérebro, a atividade da GPx1 é maior que a da catalase (Marklund *et al*., 1982). Dessa forma, como a catalase é predominantemente expressa nos peroxissomas, enquanto que a GPx1 é encontrada no citosol e mitocôndria, onde uma grande quantidade de superóxido é gerada, a GPx pode ser mais importante que a catalase na remoção do peróxido de hidrogênio no SNC (Schreibelt *et al*., 2007). O esquema simplificado da produção e remoção de espécies reativas pelas enzimas antioxidantes celulares está ilustrado na Figura 3.

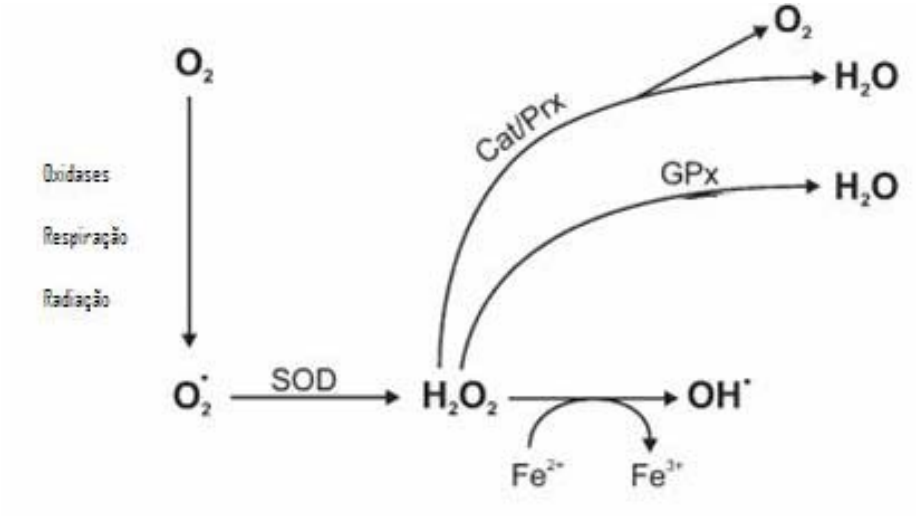


Figura 3: Esquema simplificado da produção e remoção de espécies reativas pelas enzimas antioxidantes celulares (adaptado de Maher & Schubert, 2000).

2.4 Antioxidantes e doenças neurodegenerativas

Os antioxidantes neuroprotetores são considerados uma abordagem promissora para abrandar a progressão e limitar a extensão da perda de células neuronais no caso das doenças neurodegenerativas (Moosmann & Behl, 2002).

Diferentes tipos de compostos com ações diferenciadas são a base para o desenvolvimento de drogas com potencial farmacológico antioxidante. As formas de ação podem ser através da inibição da formação de radicais livres, remoção química direta não-enzimática de radicais livres gerados através de diversas fontes e/ou detoxificação enzimática da acumulação de EROs (Moosmann & Behl, 2002).

Muitos removedores de EROs tem sido estudados em vários modelos experimentais de morte celular neuronal *in vivo* e *in vitro*. A vitamina E é um composto monofenólico e atua como um antioxidante direto, sendo que por possuir um grupo hidroxil em sua estrutura química, torna-se capaz de doar um hidrogênio para o elétron desemparelhado das EROs. Especificamente na doença de Alzheimer, a vitamina E previne a acumulação de metabólitos oxidativos induzidos pelo peptídeo β-amilóide (principal marcador da doença de Alzheimer) e dessa forma previne a neurotoxicidade deste peptídeo *in vitro*. A vitamina E também bloqueia a reação de oxidação lipídica (Behl *et al*., 1992; Behl *et al*., 1994; Harris *et al*., 1995).

A selegilina (L-defrenil) é um inibidor da mono-amino oxidase-B usada no tratamento da doença de Parkinson. Seu uso tem sido sugerido no tratamento da doença de Alzheimer, como um neuroprotetor, com ação antioxidante e geradora de óxido nítrico, que, neste caso, possui ação benéfica, caracterizando-se como um potente vasodilatador, particularmente nos vasos sangüíneos cerebrais (Thomas, 2000). Dessa forma, a mesma pode proteger o endotélio vascular dos efeitos tóxicos do peptídeo β-amilóide (Sano *et al*., 1997).

Estudos demonstram que o estrogênio possui ação antioxidante, protegendo neurônios dos efeitos tóxicos do peptídeo β-amilóide (Jaffe *et al*., 1994). Também tem sido relatado que este melhora o fluxo sangüíneo cerebral e aumenta os níveis de acetilcolina no hipocampo e encéfalo (Funk *et al*., 1991).

Alguns estudos demonstram que a melatonina (5-metoxi-*N*-acetiltriptamina), um hormônio encontrado nos organismos vivos em níveis que variam com o ciclo diário (Caniato *et al*., 2003), possui potencial antioxidante (Hardeland, 2005) com particular papel na proteção do DNA nuclear e mitocondrial (Reiter *et al*., 2001). Ao contrário dos antioxidantes clássicos, a melatonina exerce vários efeitos adicionais, que contribuem direta ou indiretamente para a redução dos radicais livres, e algumas destas ações são particularmente relevantes/específicos para o cérebro. Especificamente no cérebro, a melatonina contribui indiretamente para evitar a formação de radicais devido a várias ações que são muitas vezes negligenciadas, mas que podem ser muito relevantes. Tal hormônio é conhecido por exercer pronunciado efeito antiexcitatório e antiexcitotóxico, associados com a inibição do influxo de cálcio e com a liberação de NO e, conseqüentemente, com a prevenção do aumento da geração de radicais livres, dependentes da excitação (Hardeland, 2005). Especificamente na doença de Alzheimer, tem sido atribuído a melatonina efeitos antagônicos sobre o peptídeo β-amilóide (Hardeland, 2005).

O *trans*-resveratrol é um composto de reconhecida atividade biológica, tais como antiinflamatória e anticarcinogênica. Tem sido associado ao *trans*-resveratrol (trans-3,4',5-trihidroxistilbeno) efeito protetor frente a lesão cerebral isquêmica (Huang *et al*., 2002; Wang *et al*., 2002). Além disso, tem sido atribuído ao mesmo o papel protetor contra o dano e morte celular em culturas neuronais causados pelo peptídeo β-amilóide, NO e lipoproteínas oxidadas (Savaskan *et al*., 2003; Han *et al*., 2004). Inúmeros estudos demonstram a ação neuroprotetora do resveratrol, sendo

eficaz na remoção de radicais hidroxiperoxil, hidroxil e ânion superóxido (Bastianetto *et al*., 2000; Gupta *et al*., 2002; Sinha *et al*., 2002).

Uma série de estudos recentes tem descrito o efeito benéfico do ginseng e seus principais componentes, ginsengnosídeos, em alguns modelos de doença neurodegenerativa. Especial interesse tem sido dado a Doença de Parkinson (PD) em modelos *in vivo* ou *in vitro*. Num estudo com um modelo *in vivo*, foi relatado que a administração prolongada de extrato de ginseng G115 protegeu contra os efeitos neurotóxicos do agente indutor do parkinsonismo (1-metil-4-fenil-1,2,3,6-tetrahidropiridina) e do seu metabolito ativo 1 -metil-4-fenilpiridinium em roedores (Van Kampen *et al*., 2003).

2.5 Amnésia e escopolamina

A escopolamina é um fármaco antagonista dos receptores muscarínicos, definida como uma substância anticolinérgica. Atua impedindo a passagem de determinados impulsos nervosos ao sistema nervoso central (SNC) pela inibição da ação do neurotransmissor acetilcolina. A acetilcolina possui dois tipos de receptores: nicotínicos e muscarínicos. Os receptores muscarínicos pertencem à família de receptores acoplados a proteína G (Rang et al., 2004). A clonagem gênica revelou a existência de cinco subtipos de receptores muscarínicos: M1, M2, M3, M4 e M5 encontrados principalmente no SNC e periférico. Esses receptores atuam como mediadores de efeitos excitatórios e na atividade parassimpática pós-ganglionar (principalmente coração, musculatura lisa e glândulas). Os receptores M1, M3 e M5 atuam através da via do inositol fosfato, enquanto os M2 e M4 atuam ao inibir a enzima adenilato ciclase (Rang *et al*., 2004).

O sistema colinérgico tem importante papel nos processos de formação da memória e há evidências tanto em animais como em humanos, de que o aprendizado e memória podem ser modificados por drogas que afetam a função colinérgica central (Yamazaki *et al*., 2005). A memória é uma função do sistema nervoso e compreende três processos distintos: aquisição, consolidação e evocação. As principais estruturas envolvidas nos processos de memória são: hipocampo, córtex entorrinal, córtex parietal, córtex cingulado, amígdala, estriado e cerebelo (Izquierdo, 2002).

A escopolamina é utilizada em modelos de estudo da amnésia em que objetiva-se a procura de compostos que tenham capacidade de reverter os danos causados pela hipofunção colinérgica causada pela sua administração. Fan *et al*. (2004) demonstraram o efeito protetor de um derivado marinho no déficit de aprendizagem e memória induzido por escopolamina em ratos, sugerindo que o efeito do composto foi devido a sua ação antioxidante. Em um outro estudo El-Sherbiny *et al*. (2003) demonstraram o efeito antioxidante de *Hypericum perforatum* nas alterações oxidativas induzidas por uma dose amnésica de escopolamina no cérebro de ratos.

A deficiência de memória associada à administração de escopolamina está relacionada a uma diminuição da atividade colinérgica central neuronal devido ao bloqueio dos receptores muscarínicos (Schon *et al*., 2005). No entanto, uma grande variedade de compostos tem sido eficaz na proteção da amnésia induzida por escopolamina, indicando que outros mecanismos, além do sistema colinérgico, também estão envolvidos neste modelo. Estes compostos incluem inibidores da colinesterase (Scipione *et al*., 2008), doadores de óxido nítrico (Pitsikas *et al*., 2001), antagonistas dos receptores canabinóides CB1 (Takahashi *et al*., 2005) e compostos com atividade antioxidante (Kumar *et al*., 2000; Fan *et al*., 2005) e com atividade anti-inflamatória (Howes & Houghton, 2003). Atualmente, os inibidores da acetilcolinesterase são os únicos medicamentos aprovados para o tratamento da disfunção cognitiva na doença de Alzheimer (Giacobin, 2001), mas estão começando estudos ensaios clínicos com várias drogas eficazes no modelo de amnésia induzida por escopolamina, tais como agentes antiinflamatórios e antioxidantes (Cutler & Sramek, 2001; Doraiswamy, 2002).

2.6 Derivados pirazolínicos

Os derivados pirazolínicos são drogas de origem sintética que se caracterizam por apresentar na sua estrutura um anel pirazolínico, que é um heterociclo de cinco membros, contendo dois átomos de nitrogênio nas posições 1 e 2 do anel (Figura 4). Esses compostos possuem várias atividades farmacológicas descritas, como atividade antiinflamatória, analgésica e antipirética (Borne, 1995; Gursoy *et al*., 2000), entre outras.

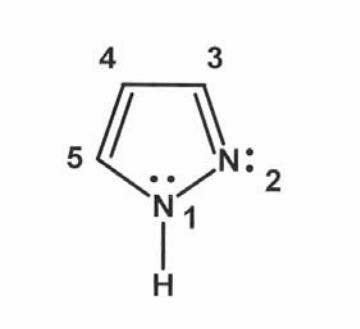


Figura 4: Anel pirazolínico

2.6.1 Histórico

A descoberta de derivados pirazolínicos data de 1884 quando foi descrito para estes atividade antipirética com a síntese da antipirina, uma droga com atividades antipirética, analgésica e anti-reumática, mas que apresentava elevada toxicidade. Mais tarde, em meados de 1940, foram sintetizadas outras pirazolidinodionas mais seguras, quando houve a descoberta da fenilbutazona, um potente antiinflamatório (Rang *et al*., 1995). Esta foi introduzida no tratamento da artrite em 1952, sendo um marco no tratamento desta doença. Todavia, ela pode ocasionar efeitos colaterais, como náuseas, vômitos e desconforto epigástrico em uma grande parcela dos pacientes. Além disso, alguns casos de agranulocitose já foram associados ao uso da fenilbutazona (Insel, 1996).

Em 1921, a dipirona foi sintetizada pelo laboratório Hoechst, sendo esta um derivado pirazolínico muito potente quanto à ação antipirética, com boa ação analgésica e fraca ação antiinflamatória (Lecannelier, 1976).

2.6.2 Ações farmacológicas dos derivados pirazolínicos

Estudos demonstraram a ação antinociceptiva dos derivados pirazolínicos no sistema nervoso central, particularmente na medula espinhal (Yaksh & Hammond, 1982; Taiwo & Levine, 1988; Hernandez & Vanegas, 2001). Também foi relatado o efeito antinociceptivo da dipirona após administração subcutânea, intratecal ou intracerebroventricular no teste das contorções abdominais (Akman *et al*., 1996). Outro estudo demonstrou que infusões repetidas e controladas de dipirona em humanos aliviam a dor crônica (Márquez & Ferreira, 1987). A dipirona também inibe o edema produzido pela aplicação de capsaicina na pele (inflamação neurogênica) (Schmeltz *et al*., 2000) Lorenzetti & Ferreira (1985) demonstraram que a administração de dipirona por via intratecal, intraperitoneal ou intraplanar inibe a nocicepção produzida por injeção de prostaglandinas, de maneira dose-dependente.

A partir do surgimento da dipirona, outros compostos contendo o anel pirazol têm sido estudados. Foi demonstrado que o composto 3-(difluormetil-1-(4-metoxifenil)-5[4-(metilsufinil)fenil] pirazol – FR140423) apresentou atividade antinociceptiva e antiinflamatória, inibindo a ciclooxigenase-2, enzima da via de biossíntese das prostaglandinas (Ochi *et* al., 1999a). Souza *et al*. (2001) demonstraram que a administração subcutânea de 3-metil-5-hidroxi-5-triclorometil-4,5-diidro-1*H*-1-pirazolcarboxiamida (MPCA) induz antinocicepção nas fases neurogênica e inflamatória do teste da formalina. Em 2004, Godoy *et al*. mostraram que o MPCA e seu análogo 3-fenil-5-hidroxi-5-triclorometil-4,5-diidro-1*H*-1-pirazolcarboxiamida (FPCA) apresentam efeito antinociceptivo no teste das contorções abdominais. Além disso, o MPCA e FPCA revertem a febre induzida por lipopolissacarídeo, quando administrados subcutaneamente (s.c.) ou intracerebroventricularmente (i.c.v.) a camundongos (Souza *et al*., 2002).

Em 2004, Tabarelli *et al*. demonstraram que outros dois derivados pirazolínicos (3-etoximetil-5-etoxicarbonil-1*H*-1-metilpirazol e 3-etoximetil-5-etoxicarbonil-1*H*-1-fenilpirazol) apresentam ação antinociceptiva num teste com estímulo nocivo térmico; e ainda que o efeito antinociceptivo do último envolve a

participação de mecanismo opióide. Outro estudo demonstrou atividade antinociceptiva de um derivado pirazol-tiazol, mostrando que esta ação ocorre de maneira dose dependente no teste das contorções abdominais induzidas pelo ácido acético (Prokopp *et al*., 2004).

Outros estudos comprovam que derivados pirazolínicos possuem atividade antimicrobiana, destacando que sua concentração inibitória mínima (MIC) é muito menor que para drogas padrão (Akbas *et al*., 2005).

Em 2006, Cunico *et al*. demonstraram a atividade antimalárica de uma série de análogos da4-(5-trifluorometil-1*H*-pirazol-1-il)-cloroquina, sendo que alguns análogos tiveram IC_{50} (concentração inibitória necessária para inibir o crescimento de 50% dos parasitos) de cerca de 2 µg/mL.

Estudos têm comprovado que compostos pirazolínicos podem apresentar atividade antioxidante, sendo capazes de remover ou evitar a formação de espécies reativas e prevenir danos oxidativos. Os mecanismos pelos quais esses compostos teriam atividade antioxidante seriam diretos ou indiretos (inibição de sistemas enzimáticos). No caso do mecanismo direto, foi demonstrado que compostos como 3,5-diaril-pirazolinas e pirazóis são capazes de inibir a oxidação de LDL *in vitro*, sendo que um destes compostos apresentou uma potência seis vezes maior que o probucol, um antioxidante sintético (Jeong *et al*., 2004). Pelo mecanismo indireto, diversos estudos têm demonstrado que esses compostos pirazolínicos são inibidores da ciclooxigenase e da lipooxigenase (Argentieri *et al*., 1994), reduzindo a formação de radicais livres por essas enzimas e atuando como antiinflamatórios. Outro estudo demonstrou que compostos como dipirona e aminopirina foram capazes de remover espécies reativas de oxigênio, como os radicais hidroxil e peroxil e o ácido hipocloroso (HOCl) originados a partir de um processo inflamatório (Costa *et al*., 2006).

As terapias antioxidantes podem ser uma alternativa para controlar as desordens associadas aos danos oxidativos, como por exemplo na demência (Ancelin *et al*., 2007)

3 RESULTADOS

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de dois manuscritos apresentados a seguir. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios manuscritos. O manuscrito 1 está disposto na versão da Revista Neurochemical Research. O manuscrito 2 está em fase final de revisão pelos autores.

3.1 Manuscrito 1

ANTIOXIDANT POTENTIAL OF NEW PYRAZOLINE DERIVATIVES TO PREVENT BRAIN OXIDATIVE DAMAGE

Manuscrito submetido à Revista *Basic and Clinical Pharmacology & Toxicology*

Antioxidant Potential of New Pyrazoline Derivatives to Prevent Brain Oxidative Damage

Daniele M. Martins[1], Bruna G. Torres[2], Patrícia R. Spohr[2], Pablo Machado[3], Helio G. Bonacorso[1,3], Nilo Zanatta[1,3], Marcos A. P. Martins[1,3], Tatiana Emanuelli[1,2,*]

[1]Graduate Program on Pharmacology, Center of Health Sciences, Federal University of Santa Maria, 97105-900 Santa Maria, RS, Brazil

[2]Integrated Center of Laboratory Analysis Development (NIDAL), Department of Alimentary Technology and Sciences, Center of Rural Sciences, Federal University of Santa Maria, 97105-900 Santa Maria, RS, Brazil

[3]Department of Chemistry, Center of Nature and Exact Sciences, Federal University of Santa Maria, 97105-900 Santa Maria, RS, Brazil

[*]Corresponding author:
Tatiana Emanuelli
Integrated Center of Laboratory Analysis Development (NIDAL)
Department of Alimentary Technology and Science
Center of Rural Sciences
Federal University of Santa Maria
Campus – Camobi, 97105-900
Santa Maria, RS - Brazil
Tel.: +55 55 3220 8547; fax: +55 55 3220 8353.
E-mail address: tatiemanuelli@smail.ufsm.br (T. Emanuelli).

Running title: Antioxidant activity of pyrazoline dervatives

Abstract: The antioxidant capacity of a series of six novel synthetic pyrazoline derivatives **(1)** 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-carbaldehyde-pyrazole, (**2**) 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-1-acetyl-pyrazole, (**3**) 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-carboxyamide-pyrazole, (**4**) 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-1-benzoyl-pyrazole, (**5**) 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-1-(2-hydroxybenzoyl)-pyrazole and (**6**) 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-1-(4-methoxybenzoyl)-pyrazol) was evaluated as the capacity of compounds to transfer an hydrogen atom (protection against brain lipid peroxidation and glutathione oxidation) and their capacity to transfer a single electron (FRAP and DPPH assays). Compound 5 had the highest free radical scavenging capacity in the DPPH assay, while compound 2 had the highest FRAP value ($p<0.05$). Only compounds 1, 4 and 5 protected against lipid peroxidation in rat brain homogenate. However, compound 5 was the most effective to prevent basal and iron-, SNP- and H_2O_2-stimulated lipid peroxidation ($IC_{50}<15$ μM) and the only one effective to block GSH oxidation mediated by H_2O_2 (at 150 μM). Our results indicate that compound 5 has the greatest potential to prevent brain oxidative damage.

Reactive oxygen species (ROS) are currently produced during cellular metabolism. Superoxide anion (O_2•-) and hydrogen peroxide (H_2O_2) formed in the mitochondria during respiration [1] may lead to the production of the highly toxic hydroxyl radical (•OH) through a metal ion-catalyzed reaction. ROS have great reactivity and may cause lipid, protein and DNA oxidation if not counteracted by endogenous antioxidant defences. Therefore, either an overproduction of ROS or a deficiency of enzymatic or non-enzymatic antioxidants may lead to oxidative stress [1].

Brain is particularly vulnerable to oxidative damage because of its high oxygen utilization, its high content of oxidizable polyunsaturated fatty acids, and the presence of redox-active metals (Cu, Fe) [2]. Accordingly, oxidative stress has been implicated in a number of neurodegenerative disorders including Alzheimer and Parkinson diseases and amyotrophic lateral sclerosis [3].

Glutathione is an important cellular antioxidant, which plays multiple roles in the nervous system including free radical scavenger, redox modulator of ionotropic receptor activity, and possible neurotransmitter [4]. It is a substrate for the detoxifying enzymes like glutathione peroxidase and glutathione reductase [4].

Sodium nitroprusside (SNP) is a vasodilator drug that releases cyanide and nitric oxide [5,6]. Although nitric oxide and/or cyanide release are currently pointed as responsible for SNP pro-oxidative effects, there is evidence that SNP-induced oxidative brain injury is actually mediated by •OH radicals generated by the iron moiety of SNP [7]. Glutathione and other thiols are among the endogenous compounds capable of reducing SNP and affecting NO release [7].

Although iron is an essential metal for cellular metabolism, it may be a potential source of ROS and is associated to oxidative stress and neurotoxicity [8]. Excessive iron deposition has been observed in the central nervous system in a number of neurodegenerative

diseases [9]. Iron accumulation and oxidative stress precede Alzheimer´s disease-associated lesions and has been suggested to be involved in the pathogenesis of such disorder [8].

Due to the important role of ROS in the pathophysiology of various neurodegenerative disorders, and because clinically effective drugs for the treatment of these disorders are scarce, there is growing interest for the development of novel antioxidant compounds [10,11,12,13,14].

The pyrazole ring is a heterocyclic of five members, with two contiguous nitrogen atoms and three carbon atoms (Fig. 1). Various synthetic pyrazole compounds have potential antipyretic [15], and antinociceptive properties [16,17,18]. Besides, it has been observed that some synthetic heterocyclic compounds with a pyrazole ring have antioxidant activity by inhibiting neutrophil oxidative burst [19].

In the current study, we investigated the antioxidant capacity of a series of six novel pyrazole derivatives using assays that evaluate the capacity of compound to transfer an hydrogen atom (protection against lipid peroxidation and glutathione oxidation) and assays that evaluate the capacity of compounds to transfer a single electron (FRAP and DPPH assays) [20]. Besides, the capacity of compounds to direct scavenging H_2O_2 and superoxide anion radical was also evaluated. Prooxidant agents that play important role in oxidative damage associated to neurodegenerative disorders (H_2O_2 and iron) or generate oxidant species that are involved in such damage (SNP) were used to stimulate lipid peroxidation in order to get further insight into the potential antioxidant capacity of compounds in the brain. The pyrazole compounds evaluated had the same basic structure (5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-pyrazoles), but different substituents in the position 1 of the pyrazole ring. Therefore, we also obtained some structure-activity relationship information for these compounds.

Materials and Methods

The present study was approved by the Ethics and Animal Welfare Committee of Federal University of Santa Maria, RS, Brazil.

Chemicals. Glacial acetic acid, hydrochloric acid, ethanol, methanol, K_2HPO_4, KH_2PO_4, 5,5'-dithiobis-(2-nitrobenzoic acid) and hydrogen peroxide were obtained from Merck (Rio de Janeiro, Brasil). Tris (hydroxymethyl) aminomethane, thiobarbituric acid, reduced glutathione, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-trypyridyl-s-triazine (TPTZ) and trolox were obtained from Sigma Chemical Co. (St. Louis, MO, USA). $FeCl_3$ was obtained from Synth (São Paulo, Brazil). $FeCl_2$, sodium acetate, trichloroacetic acid, n-butyl alcohol and SNP were obtained from Vetec (São Paulo, Brazil).

Pyrazole compounds. The novel 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-pyrazoles, **(1)** 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-carbaldehyde-pyrazole, (**2**) 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-1-acetyl-pyrazole, (**3**) 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-carboxyamide-pyrazole, (**4**) 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-1-benzoyl-pyrazole, (**5**) 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-1-(2-hydroxybenzoyl)-pyrazole and (**6**) 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-1-(4-methoxybenzoyl)-pyrazole (Fig. 1) were synthesized from cyclocondensation reaction of the appropriate hydrazide with 1,1,1-trifluoro-4-methoxy-3-penten-2-one according to previously reported procedures [21,22,23]. Analysis of the ^{1}H and ^{13}C NMR and mass spectra showed analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of compounds (98-100%) was determined by GC/MS. Solutions of the pyrazole compounds (0.5, 1 or 5 mM) were prepared in ethanol p.a.

98% and used in all experiments for evaluation of their antioxidant capacity. The same amount of ethanol 98% was used as control in all experiments.

Protection against lipid peroxidation. Lipid peroxidation was assessed in brain homogenate from adult male Wistar rats (150-200 g) from our own breeding colony that were maintained at 25°C, on a 12 h light/12h dark cycle, with water and food *ad libitum*. Rats were decapitated under mild ether anesthesia and the forebrain was rapidly dissected and homogenized in 50 mM Tris-HCl, pH 7.5 (1/10, w/v). The homogenate was centrifuged for 20 min at 2,000 *x g* to yield a low-speed supernatant that was used for lipid peroxidation determination. This supernatant (350 µL) was incubated at 37°C for 1h in the presence or absence of 50 µM $FeCl_2$, 1 mM H_2O_2 or 5 µM SNP and pyrazole compounds (0, 15, 30 or 150 µM) in a final volume of 500 µL of Tris-HCl 50 mM. After this incubation the amount of thiobarbituric acid reactive substances formed was determined as described by Ohkawa et al. [24].

IC_{50} calculation. IC_{50} for lipid peroxidation (concentration inhibiting 50% of lipid peroxidation) was determined by non-linear regression analysis using GraphPad Prism Program version 4.0. IC_{50} values for lipid peroxidation stimulated by iron, SNP or H_2O_2 were calculated as the concentration of pyrazole compound to inhibit 50% of the increase of lipid peroxidation induced by the prooxidant agent, i.e. basal lipid peroxidation was subtracted from the stimulated lipid peroxidation values.

Ferric-reducing antioxidant power (FRAP) assay. A modified method of Benzie and Strain [25] was used for FRAP assays. Ferric-TPTZ solution was prepared by mixing 2.5 ml 10 mM TPTZ solution in 40 mM HCl, 2.5 ml 20 mM $FeCl_3.6H_2O$ and 25 ml of 0.3 M acetate buffer at pH 3.6. Sample (40 µl of 0.5 mM solution) was mixed with 1.2 ml of ferric-TPTZ reagent

and incubated at 37°C during 15 min. The absorbance of the colored complex formed with Fe^{+2} and TPTZ was determined at 593 nm. Trolox was used as standard for the calibration curve and results were expressed as trolox equivalents (mol trolox/mol of compound).

DPPH radical scavenging assay. A stable solution of DPPH was used for determination of total antioxidant capacity of compounds with Brand-Williams *et al.* [26] modified method. DPPH solution (0.24 mg/ml) was previously diluted until 1.10±0.02 absorbance at 517 nm was obtained. Compounds (50 µl of a 0.5 mM solution) were mixed with 1.95 ml diluted methanolic DPPH solution. The antiradical power of the different compounds was determined by measuring the decrease of DPPH absorbance after 24 hours in the dark against a blank. Thus, the addition of an antioxidant results in a decrease of absorbance proportional to the antioxidant activity of the compound itself. Trolox was used as standard for the calibration curve and results were expressed as trolox equivalents (mol trolox/mol of pyrazole compound).

Protection against glutathione oxidation. The capacity of the compounds to prevent glutathione (GSH) oxidation was evaluated in the absence or presence of H_2O_2 by measuring the disappearance of –SH groups from reduced glutathione. Reduced –SH groups of GSH were quantified as described by Ellman [27] at 0, 30, 60, 90 and 120 min after GSH (1mM) addition to a reaction mixture containing 200 mM potassium phosphate buffer, pH 6.4, pyrazole compounds (0, 15, 30 or 150 µM) and hydrogen peroxide (0 or 0.25 mM) at 39°C. Controls containing pyrazole compounds with no glutathione were run in order to verify a possible absorbance of these compounds at the wavelength used to assess glutathione oxidation (412 nm). None of the pyrazole compounds had any measurable absorbance at this wavelength in the concentration range evaluated.

Evaluation of H_2O_2 scavenging capacity. The ability of pyrazole compounds to scavenge H_2O_2 was evaluated by the decreasing of H_2O_2 absorbance at 240 nm in a medium containing 50 mM phosphate buffer, pH 7.0 and 17 mM H_2O_2 at 25^{o}C [28].

Evaluation of superoxide anion radical scavenging capacity. The ability of pyrazole compounds to inhibit the auto-oxidation of epinephrine to adrenochrome, which is mediated by superoxide anions, was assessed at 480 nm. Reaction assay contained 50 mM glycine buffer, pH 10.2, and 1 mM epinephrine at 30°C [29].

Statistical analysis. Data on the *in vitro* effects of pyrazole compounds on lipid peroxidation were analyzed by two-way analysis of variance (ANOVA) (4 pyrazole concentrations x 4 incubation conditions). Data on the rate of GSH oxidation were analyzed by two-way ANOVA (4 pyrazole concentrations x 5 incubation times) considering the time variable as a repeated measure. Data on the FRAP and DPPH assays were analyzed by one-way ANOVA (6 pyrazole compounds). Results were post hoc compared using Duncan's multiple range test when necessary. Results with $p<0.05$ were considered significant.

Results

Lipid peroxidation was assessed in rat brain homogenate. Fe and SNP significantly increased lipid peroxidation in rat brain, while H_2O_2 caused a small but not significant increase in lipid peroxidation when compared to basal values (Fig. 2). Pyrazole compounds 2, 3 and 6 had no effect on lipid peroxidation in rat brain homogenate (data not shown). At 150 μM pyrazole compound 1 did reduce iron- and SNP-stimulated lipid peroxidation ($P<0.05$; Fig. 2A). However, this compound had no effect on basal or H_2O_2-stimulated lipid

peroxidation. Pyrazole compound 4 was effective in reducing basal, iron- and SNP-stimulated lipid peroxidation from 15 μM onwards ($P<0.05$; Fig. 2B). However, this compound had no effect on H_2O_2-stimulated lipid peroxidation. Compound 5 was the most effective to reduce lipid peroxidation in all conditions tested. It did reduce basal, iron-, SNP- and H_2O_2-stimulated lipid peroxidation from 15 μM onwards ($P<0.05$; Fig. 2C).

Compound 5 had lower IC_{50} values for basal (3.1 μM) and H_2O_2-stimulated lipid peroxidation (< 15 μM) than compounds 1 (> 150μM and > 150 μM, respectively) and 4 (> 150 μM and > 150 μM, respectively). However, compounds 1, 4 and 5 had similar IC_{50} values for iron- (21.5, 6.0 and 7.7 μM, respectively) and SNP-stimulated lipid peroxidation (9.2, < 15 μM and < 15 μM). IC_{50} values for the pyrazole compounds 2, 3 and 6 were not determined because no significant inhibition of lipid peroxidation was observed at concentrations as high as 150 μM. According to the IC_{50} values, compounds 1 and 4 were more potent to inhibit iron- and SNP-stimulated lipid peroxidation, when compared to basal and H_2O_2-stimulated lipid peroxidation. In contrast, compound 5 had a similar potency to inhibit lipid peroxidation in all conditions evaluated (basal and stimulated lipid peroxidation).

The antioxidant capacity of the six novel pyrazole compounds assessed using the FRAP and DPPH assays was expressed as equivalents of the standard antioxidant trolox, which is a hydrosoluble analogous of vitamin E. All compounds had ferric-reducing power, but compound 2 had FRAP values significantly higher than the other compounds and compound 6 had FRAP values significantly higher than compounds 1 and 3 (Table I). Compound 5 was the most effective to scavenge DPPH radical when compared to the other compounds ($P<0.05$; Table II). Compounds 1 to 4 had a very low DPPH scavenging capacity, while compound 6 had no detectable DPPH scavenging capacity (Table II).

The ability of pyrazole compounds to prevent spontaneous or H_2O_2-stimulated glutathione oxidation was also evaluated (Fig. 3). We observed no significant spontaneous

glutathione oxidation during our incubation assay (Fig. 3A). However, a significant glutathione oxidation was observed after 120 min incubation in the presence of H_2O_2 (Fig. 3B). None of the pyrazole compounds evaluated prevented the oxidation of glutathione (data not shown), with the exception of compound 5 (Fig. 3). Compound 5 (150 μM) significantly increased the amount of reduced glutathione after 90 min of incubation in the absence of H_2O_2 ($P<0.05$, Fig. 3A). Although we used reduced glutathione in this assay, a small amount of GSH was probably oxidized during handling of solutions before starting incubation. Probably this previously oxidized glutathione was the one reduced by pyrazole compound 5 during incubation in the absence of H_2O_2. Also, compound 5 (150 μM) completely prevented H_2O_2-stimulated GSH oxidation (Fig. 3B).

In order to get further insight into the antioxidant mechanism we also evaluated the ability of compounds to scavenge H_2O_2 and superoxide anion radical. None of the evaluated compounds were effective to remove either H_2O_2 or superoxide anion radical (data not shown).

Discussion

In this study, the antioxidant capacity of six novel pyrazole compounds was evaluated. Only compounds 1, 4 and 5 had some protective effect against brain lipid peroxidation. The most effective compound was 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-1-(2-hydroxybenzoyl)-pyrazole (compound 5), with IC_{50} values < 15μM for all conditions tested, indicating that binding an hydroxybenzoyl group to the basic structure of the pyrazole compound increases antioxidant activity. This finding is in accordance with previous studies showing that the hydroxybenzoyl group has a key role as free radical scavenger in other classes of compounds [30,31]. In contrast, methyl, amine or methoxybenzoyl groups bound to the basic structure of the pyrazole compound (compounds 2, 3 and 6) decreased lipid

antioxidant activity as compared to the hydrogen substituted compound (compound 1), while a benzoyl group (compound 4) had no effect.

The protective effect of the pyrazole compounds tested was different depending on the agent used to induce lipid peroxidation. Compounds 1 and 4 were effective to prevent iron- and SNP-stimulated lipid peroxidation, while compound 5 was effective to prevent lipid peroxidation in all conditions evaluated (basal and stimulated). The cytotoxicity of SNP may be mediated by nitric oxide (NO) production [6] and also by •OH radicals generated by the iron moiety of SNP after NO release [7]. Iron triggers the generation of highly reactive oxygen species, such as •OH radicals via the Fenton reaction, which depends on H_2O_2 [32], or the Haber-Weiss reaction, which depends on H_2O_2 and superoxide anion radical. In sequence, iron and •OH radicals may initiate a lipid peroxidation chain reaction and oxidative brain injury [33,34]. None of the evaluated pyrazole compounds had direct superoxide anion radical or H_2O_2 scavenging properties. However, the high DPPH scavenging power of compound 5 suggests that it could be able to directly remove free radicals important for lipid peroxidation. It is known that phenolic compounds play a key role as antioxidants due to the presence of hydroxyl substituents in their aromatic structure, which enables them to scavenge free radicals [35]. Hence, the hydroxybenzoyl substituent of compound 5 could be involved in transferring labile electrons to the DPPH radical. Although the DPPH assay involves an hydrogen atom transfer reaction, Foti et al. [36] provided evidence that it in fact behaves like an electron transfer reaction, since the rate-determining step consists of a fast electron transfer process from phenolic compounds to DPPH. In agreement with the highest DPPH scavenging power, compound 5 was effective to prevent basal or stimulated lipid peroxidation in rat brain.

In addition, compound 5 was the only compound that was able to prevent GSH oxidation induced by H_2O_2. This finding suggests that compound 5 could have H_2O_2 scavenging activity. However, neither compound 5 nor any of the other pyrazole compounds

evaluated were able to directly remove H_2O_2. Compound 5 probably protected against glutathione oxidation by directly reducing this compound, since it was able to reduce previously oxidized glutathione (incubation in the absence of H_2O_2).

In the FRAP assay the antioxidant capacity is measured as a reducing ability, i.e. the ability to reduce TPTZ-Fe(III) complex to TPTZ-Fe(II) complex [37]. Among the compounds evaluated compound 2 had FRAP values significantly higher than the other compounds and compound 6 had FRAP values significantly higher than compounds 1 and 3, suggesting that compounds 2 and 6 are good reducing agents. However, the reducing power of these compounds was not confirmed in the DPPH assay that also evaluates the capacity of compounds to transfer a single electron. Moreover, these compounds had no antioxidant activity either against lipid peroxidation or glutathione oxidation. On the other hand, compound 5, that was effective to prevent both lipid peroxidation and glutathione oxidation, was among the four compounds with lowest FRAP activity. These results indicate that FRAP assay had no correlation with antioxidant activity in the other models investigated.

Among the six pyrazole compounds evaluated the most effective antioxidant in the *in vitro* models assessed was 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-1-(2-hydroxybenzoyl)-pyrazole (compound 5). Results of DPPH assay demonstrated that compound 5 has a free radical scavenging capacity [37]. Compound 5 was also effective to prevent basal and iron-, SNP- or H_2O_2-stimulated lipid peroxidation in rat brain homogenate and the only one effective to block GSH oxidation mediated by H_2O_2.

Membrane lipid peroxidation occurs in various neurodegenerative disorders including amyotrophic lateral sclerosis, Alzheimer's (AD), Parkinson's (PD) and Huntington's disease [37]. Although different genetic and environmental factors seem to initiate membrane-associated oxidative stress in each disorder, the involvement of redox-active metals, especially iron and copper, is a common hallmark in these disorders [38]. Levels of iron are

increased in vulnerable neuronal populations in AD and PD, and agents that chelate iron and/or copper are beneficial in animal models of AD and PD [38]. Also, antioxidants that suppress membrane lipid peroxidation, like vitamin E, protect neurons in experimental models of neurodegenerative disorders [39]. Therefore, the antioxidant effect of compound 5 against lipid peroxidation, including that stimulated by iron seems to be interesting for the treatment of neurodegenerative disorders. Other studies are necessary to evaluate the therapeutic potential of this compound in models of neurodegenerative disorders *in vivo*.

Acknowledgements

Work supported by PRONEX-FAPERGS-CNPq (grant 0408660 to M.A.P. Martins). D.M. Martins is the recipient of CAPES master degree Fellowship and T.E. is the recipient of CNPq research Fellowship (proc. 306432/2007-2). The authors thank Juliana da Rocha V. Torres for technical assistance in some TBARS assays.

References

1 Adam-Vizi V. Production of reactive oxygen species in brain mitochondria: contribution by electron transport chain and nonelectron transport chain sources. Antioxid Redox Signal 2005;**7**:1140-49.

2 Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J. Free radical and antioxidants in normal physiological functions and human disease. Int J Biochem Cell B 2007;**39**:44-84.

3 Cadet JL, Brannock C. Free radicals and the pathobiology of brain dopamine systems. Neurochem Res 1998; **32**:117-31.

4 Genovese T, Mazzon E, Esposito E, Muià C, Di Paola R, Di Bella, P et al. Role of endogenous glutathione in the secondary damage in experimental spinal cord injury in mice. Neurosci Lett 2007; **423**:41-6.

5 Arnold WP, Longneeker DE, Epstein RM. Photodegradation of sodium nitroprusside: Biologic activity and cyanide release. Anesthesiology 1984; **61**:254-60.

6 Bates JN, Baker MT, Guerra R, Harrison DG. Nitric oxide generation from nitroprusside by vascular tissue. Biochem Pharmacol 1991;**42**:S157-S65.

7 Rauhala P, Khaldi A, Parameswarannay K, Chiueh CC. Apparent role of hydroxyl radicals in oxidative brain injury induced by sodium nitroprusside. Free Radical Bio Med 1998; **24**:1065-73.

8 Castellani RJ, Moreira PI, Liu G, Dobson J, Perry G, Smith MA et al. Iron: the redox-active center of oxidative stress in Alzheimer disease. Neurochem Res 2007;**32**:1640-45.

9 Roy CN, Andrews NC. Recent advances in disorders of iron metabolism: mutations, mechanisms and modifiers. Hum Mol Genet 2001:**10**:2181–86.

10 Rao AV, Balachandran B. Role of oxidative stress and antioxidants in neurodegenerative diseases. Nutr Neurosci 2002;**5**:291-309.

11 Facheris M, Beretta S, Ferrarese C. Peripheral markers of oxidative stress and excitotoxicity in neurodegenerative disorders: Tools for diagnosis and therapy? J Alz Dis 2004;**6**:177-84.

12 Naoi M, Maruyama W, Shamoto-Nagai M, Yi H, Akao Y, Tanaka, M. Oxidative stress in mitochondria: decision to survival and death of neurons in neurodegenerative disorders. Mol. Neurobiol 2005;**31**:81-93.

13 Reynolds A, Laurie C, Mosley RL, Gendelman HE. Oxidative stress and the pathogenesis of neurodegenerative disorders. Int Rev Neurobiol 2007;**82**:297-325.

14 Anderson CM, Halberg A, Brattsand R, Engman L, Persson J, Moldeus P et al. Diaryl telurides as inhibitors of lipid peroxidation in biological and chemical systems. Free Radical Res 1993;**20**:401-10.

15 Souza FR, Souza VT, Ratzlaff V, Borges LP, Oliveira MR, Bonacorso HG et al. Hypothermic and antipyretic effects of 3-methyl- and 3-phenyl-5-hydroxy-5-trichloromethyl-4,5-dihydro-1*H*-pyrazole-1-carboxyamides in mice. Eur J Pharmacol 2002;**451**:141-47.

16 Tabarelli Z, Rubin MA, Berlese DB, Sauzem PD, Missio TP, Teirxeira MV et al. Antinociceptive effect of novel pyrazolines in mice. Braz J Med Biol Res 2004;**37** (10):1531-40.

17 Prokopp CR, Rubin MA, Sauzem PD. A pyrazolyl-thiazole derivative causes antinoception in mice. Braz J Med Biol Res 2006;**39**:795-99.

18 Godoy MCM, Fighera MR, Souza FR, Flores AE, Rubin MA, Oliveira MR et al. α_2-Adrenoceptors and 5-HT receptors mediate the antinociceptive effect of new pyrazolines, but not of dipyrone. Eur J Pharmacol 2004;496: 93-97.

19 Costa D, Marques, AP, Reis RL, Lima JLFC, Fernandes E. Inhibition of human neutrophil oxidative burst by pyrazolone derivatives. Free Radical Bio Med 2006;**40**:632-40.

20 Huang D, Ou B, Prior RL. The chemistry behind antioxidant capacity assays. J Agr Food Chem 2005;**53**:1841-56.

21 Bonacorso HG, Wentz AP, .Lorega R V, Cechinel CA, Moraes TS, Coelho HS et al. Trifluoromethyl-containing pyrazolinyl (p-toluyl) sulfones: The synthesis and structure of promising antimicrobial agents. J Fluorin Chem 2006;**127**:1066-72.

22 Martins MA.P, Beck P, Machado P, Brondani S, Moura S, Zanatta L et al. Microwave-assisted of novel 5- trichloromethyl-4,5-dihydro-1*H*-1-pyrazole methyl esters under solvent free conditions. J Braz Chem Soc 2006;**17**:408-11.

23 Bonacorso H G, Oliveira M R, Costa, MB. Regiospecific one-pot synthesis of new trifluoromethyl substituted heteroaryl pyrazolyl ketones. J Heterocycl Chem 2005;**42**: 631-37.

24 Ohkawa H, Ohishi, N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;**95**:351-58.

25 Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of ‘‘antioxidant power’’: the FRAP assay. Anal Biochem 1996;**239**:70–6.

26 Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. Lebensm- Wiss und-Technol 1995;**28**:25–30.

27 Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959;**82**:70-7.

28 Aebi H. Catalase in vitro. Meth Enzymol 1984;**105**:121-26.

29 Misra HP, Fridovich I. The role of superoxide anion in the autooxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 1972; **247**:3170–75.

30 Kefalas P, Kallithraka S, Parejo I, Makris DP. A comparative study on the in vitro antiradical efficiency and hydroxyl free radical scavenging activity in aged red wines. Food Sci Technol Int 2003;**9**:9383-87.

31 Verhagen JV, Haemen GRMM, Bast A. Nitric oxide radical scavenging by wines. J Agr Food Chem 1996;**44**:3733-34.

32 Graf E, Mahoney JR, Bryant RG, Eaton JW. Iron-catalyzed hydroxyl radical formation: Stringent requirement for free iron coordination site. J Biol Chem 1984;**259**:3620-24.

33 Halliwell B. Reactive oxygen species and the central nervous system. J Neurochem 1992;**59**:1609-23.

34 Mohanakumar KP, De Bartolomeis A, Wu RM, Yeh KJ, Sternberger LM, Peng SY et al. Ferrous-citrate complex and degeneration: evidence for free-radical formation and lipid peroxidation. Ann N Y Acad Sci 1994;**738**:392-9.

35 Villaño D, Pachón-Fernandez MS, Moyá ML. Radical scavenging ability of polyphenolic compounds towards DPPH free radical. Talanta 2007;**71**:230-5.

36 Foti MC, Daquino C, Geraci C. Electron-transfer reaction of cinnamic acids and their methyl esters with the DPPH radical in alcohol solutions. J Org Chem 2004;**69**:2309-14.

37 Huang D, Ou B, Prior RL. The chemistry behind antioxidant capacity assays. J. Agr. Food Chem 2005;**53**:1841-56.

38 Mattson MP. Metal-catalyzed disruption of membrane protein and lipid signaling in the pathogenesis of neurodegenerative disorders. Ann N Y Acad Sci 2004;**1012**:37-50.

39 Kontush A, Schekatolina S. Vitamin E in neurodegenerative disorders Alzheimer's disease. Ann NY Acad Sci 2004;**1031**:249-62.

Figure captions:

Fig. 1 Chemical structures of pyrazoline derivatives (5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-pyrazoles) evaluated.

Fig. 2 Effects of compounds 1 (5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-carbaldehyde-pyrazole, A), 4 (5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-1-benzoyl-pyrazole, B) and 5 (5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-1-(2-hydroxybenzoyl)-pyrazole, C) on spontaneous (basal), Fe (50 μM), SNP (5 μM) and H_2O_2 (1 mM)-induced TBARS production (Abs). Results are expressed as mean ± S.E.M., n=4. *Different from basal at the same concentration (P<0.05). [&]Fe and SNP were different from control (0 μM) at the same condition (P<0.05). [#]Basal, Fe and SNP were different from control (0 μM) at the same condition (P<0.05). [$]Different from control (0 μM) at the same condition (P<0.05).

Fig. 3 Effect of pyrazole compound 5 (5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-1-(2-hydroxybenzoyl)-pyrazole) on the rate of glutathione (1mM) oxidation in the absence (A) or presence (B) of H_2O_2. Sulfhydryl groups of glutathione were evaluated at 412 nm with 5,5'-dithio-bis-(2-nitrobenzoic acid). Results are the mean of three independent experiments. S.E.M. were less than 15% of respective means. *Different from control (0 μM) at the same time (P<0.05). [&]Different from the same group at time zero (P<0.05).

Table I Antioxidant capacity of new pyrazole compounds

Pyrazole compound	FRAP (mol trolox/mol of compound)	DPPH (mol trolox/mol of compound)
1	0.151±0.035 [c]	0.011±0.010 [b]
2	0.779±0.201 [a]	0.004±0.004 [b]
3	0.119±0.056 [c]	0.046±0.033 [b]
4	0.281±0.079 [b,c]	0.066±0.038 [b]
5	0.299±0.036 [b,c]	1.276±0.479 [a]
6	0.476±0.124 [b]	no results[&]

Results are the mean of five independent measurements ± S.E.M. Values are expressed as trolox equivalents. FRAP: ferric reducing antioxidant power. DPPH: 1,1-diphenyl-2-picrylhydrazyl radical scavenging assay. Values that have no common superscript letter are different by Duncan's multiple range test ($P<0.05$). [&]No DPPH scavenger capacity was detected in the assay conditions.

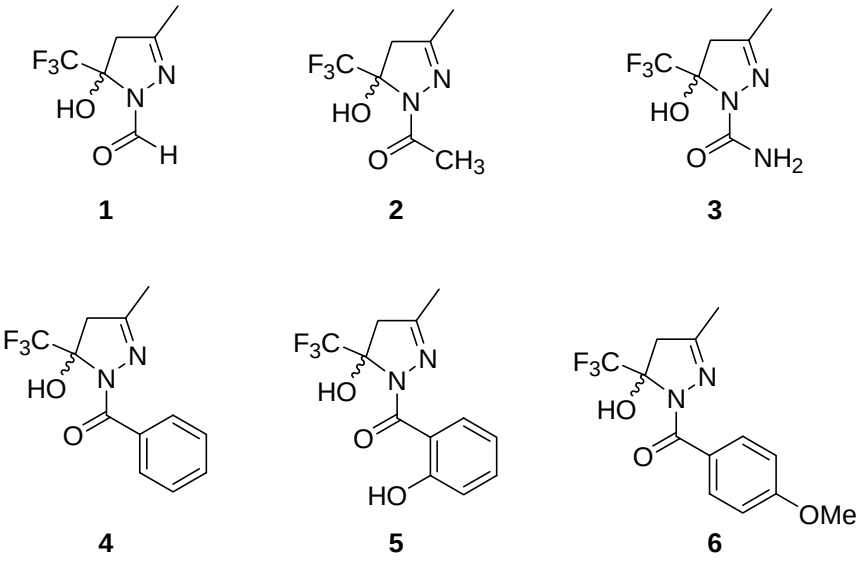

F3C
HO
N
N
O
H
1
F3C
HO
N
N
O
CH3
2
F3C
HO
N
N
O
NH2
3
F3C
HO
N
N
O
4
F3C
HO
N
N
O
HO
5
F3C
HO
N
N
O
OMe
6

Fig. 1

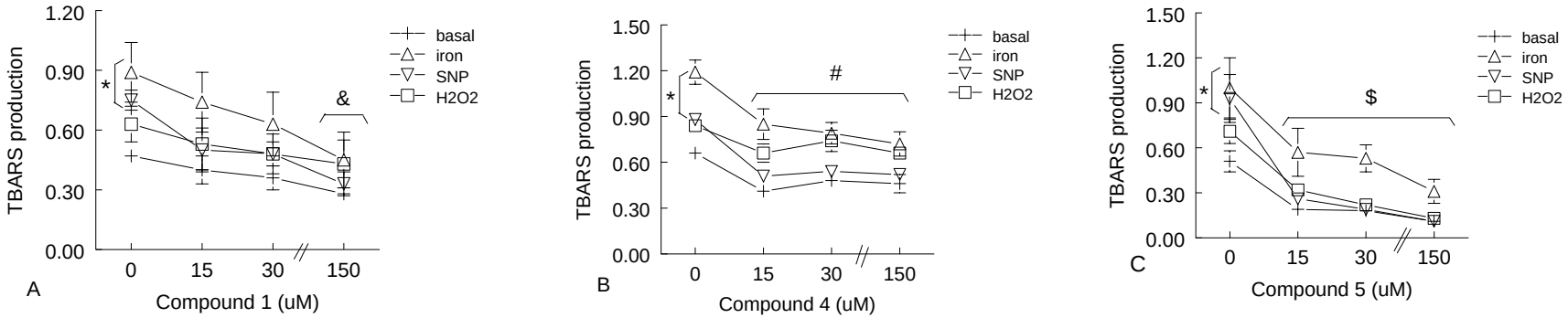
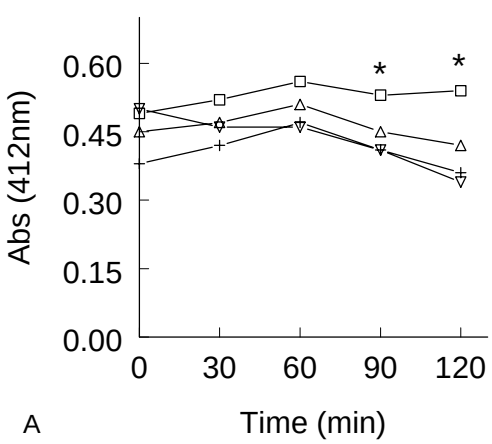
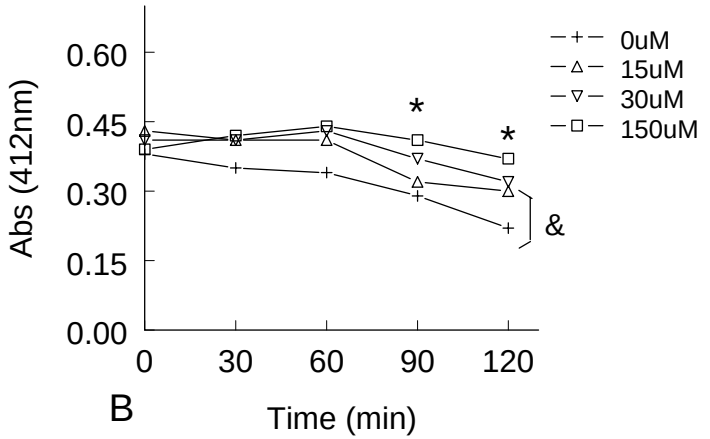

1.20
0.90
0.60
0.30
0.00
TBARS production
*
&
0
15
30
150
Compound 1 (uM)
A
basal
iron
SNP
H2O2
1.50
1.20
0.90
0.60
0.30
0.00
TBARS production
*
#
0
15
30
150
Compound 4 (uM)
B
basal
iron
SNP
H2O2
1.50
1.20
0.90
0.60
0.30
0.00
TBARS production
*
$
0
15
30
150
Compound 5 (uM)
C
basal
iron
SNP
H2O2

Fig. 2


0.60
0.45
0.30
0.15
0.00
Abs (412nm)
0
30
60
90
120
Time (min)
*
*

A


0.60
0.45
0.30
0.15
0.00
Abs (412nm)
0
30
60
90
120
Time (min)
*
*
&
0uM
15uM
30uM
150uM

B

Fig. 3

3. 2 Manuscrito 2

5-HYDROXI-3-METHYL-5-TRIFLUOROMETHYL-4,5-DIHYDRO-1H-1-(2-HYDROXYBENZOYL)-PYRAZOLE PREVENTS AMNESIA INDUCED BY SCOPOLAMINE IN RATS

Manuscrito em fase final de revisão pelo autores

5-HYDROXY-3-METHYL-5-TRIFLUOROMETHYL-4,5-DIHYDRO-1H-1-(2-HYDROXYBENZOYL)-PYRAZOLE PREVENTS AMNESIA INDUCED BY SCOPOLAMINE IN RATS

Daniele M. Martins[1], Nardeli Boufleur[2], Sabrina Somacal[2], Cristiane Casagrande Denardin[2], Pablo Machado[3], Maribel A. Rubin[1], Helio G. Bonacorso[1,3], Nilo Zanatta[1,3], Marcos A. P. Martins[1,3], Tatiana Emanuelli[1,2,*]

[1]Graduate Program on Pharmacology, Center of Health Sciences, Federal University of Santa Maria, 97105-900 Santa Maria, RS, Brazil

[2]Integrated Center of Laboratory Analysis Development (NIDAL), Department of Alimentary Technology and Sciences, Center of Rural Sciences, Federal University of Santa Maria, 97105-900 Santa Maria, RS, Brazil

[3]Department of Chemistry, Center of Nature and Exact Sciences, Federal University of Santa Maria, 97105-900 Santa Maria, RS, Brazil

[*]Corresponding author:

Tatiana Emanuelli
Integrated Center of Laboratory Analysis Development (NIDAL)
Department of Alimentary Technology and Science
Center of Rural Sciences
Federal University of Santa Maria
Campus – Camobi, 97105-900
Santa Maria, RS - Brazil
Tel.: +55 55 3220 8547; fax: +55 55 3220 8353.
E-mail address: tatiemanuelli@smail.ufsm.br (T. Emanuelli).

Abstract Alzheimer's disease (AD) is characterized by memory loss that is accompanied by degeneration of basal forebrain cortical cholinergic neurons and increased levels of markers of oxidative stress in the brain. 5-Hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1H-1-(2-hydroxybenzoyl) pyrazole was recently demonstrated to prevent brain lipid peroxidation and glutathione oxidation. This study evaluated the effect of this pyrazole compound (100, 300 or 1000 μmol/kg, i.p.) on the scopolamine-induced amnesia and oxidative stress parameters in rat brain. A step-down inhibitory avoidance apparatus was used to evaluate the effect of scopolamine (1.4 mg/g, i.p.) on memory function, following the assessments of oxidative stress biomarkers (brain thiobarbituric acid reactive substances, non-protein thiol groups, superoxide dismutase and catalase activities). Scopolamine administration 30 min before training session resulted in shorter latency to step-down during the test session as compared to control groups ($P<0.05$). Pretreatment with pyrazole compound (30 min before scopolamine) had no effect *per se* on the step-down latency. However, pyrazole compound prevented the amnesic effect of scopolamine in a bell-shaped curve (100 μmol/kg pyrazole was the most effective dose, $P<0.05$). No significant effect of scopolamine or pyrazole treatment was observed on any of the oxidative stress markers evaluated, suggesting that the protective effect of pyrazole was not related to a possible antioxidant activity. These findings suggest that this pyrazole compound could be a promising drug for the treatment of AD and should be further evaluated in other models of dementia.

1. INTRODUCTION

Various evidence demonstrate the involvement of oxidative stress in some neurodegenerative disorders including Alzheimer's disease (AD) (Cross *et al*., 1987; Smith *et al*., 1995). Over the past decade, modification of virtually all classes of biomolecules indicative of oxidative stress has been described in association with the susceptible neurons of AD, so DNA and RNA oxidation is marked by increased levels of 8-hydroxyl-2-deoxyguanosine and 8-hydroxyguanosine (Nunomura *et al*., 2001). Oxidative modification of proteins is marked by significantly elevated levels of protein carbonyl and widespread nitration of tyrosine residues (Smith *et al*., 1996; Smith *et al*., 1997). Lipid peroxidation is marked by higher levels of thiobarbituric acid reactive substances (TBARS), malondialdehyde (MDA), 4-hydroxy-2-transnonenal and isoprostane and altered phospholipid composition (Sayre *et al*., 1997; Butterfield *et al*., 2001) Accordingly, reactive oxygen species are speculated to be pathologically important in AD.

Antioxidant enzymes like superoxide dismutase (SOD) and catalase (CAT) are important in the protection against reactive oxygen species like O_2^- and H_2O_2. Enhanced expression/activity of these enzymes has been commonly used as relevant indices of brain oxidative stress (Illic *et al*., 1999). Also, the tripeptide GSH is a redox regulator that participates in the maintenance of oxidant homeostasis and in the cellular detoxification of ROS in brain cells (Cruz *et al*., 2003).

The cholinergic-neuronal system plays an important role in learning and memory in humans and animals (Van der Zee & Luiten, 1999). Accordingly, AD is characterized by memory loss that is accompanied by degeneration of basal forebrain cortical cholinergic neurons. The muscarinic antagonist scopolamine interferes with cholinergic transmission in the central nervous system and impairs learning and memory, especially the processes of learning acquisition and short-term memory. As such, scopolamine has been used to induce

amnesia in animals. This experimental model of dementia is currently used in screening anti-amnesic drugs that could be useful for the treatment of AD (Kang *et al*., 2003). The impaired cognitive function in this animal model has been recently suggested to be associated to elevated brain oxidative status (El-Sherbiny *et al*., 2003; Khalifa, 2004; Fan *et al*., 2005).

Various synthetic pyrazole compounds have potential antipyretic (Souza *et al*., 2002), and antinociceptive properties (Tabarelli *et al*., 2004; Godoy *et al*., 2004; Prokopp *et al*., 2006). Besides, it has been observed that some synthetic heterocyclic compounds with a pyrazole ring have antioxidant activity by inhibiting neutrophil oxidative burst (Costa *et al*., 2006) and preventing brain lipid peroxidation (Martins *et al*., 2008). In a study the evaluated the potential of a new series of pyrazole compounds to prevent brain oxidative damage in vitro, we observed that 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-1-(2-hydroxybenzoyl)-pyrazole (Fig. 1) was the most effective compound (Martins *et al*., 2008). Therefore, considering the involvement of oxidative stress in the dementia associated to AD, we evaluated the effect 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-1-(2-hydroxybenzoyl)-pyrazole to prevent amnesia and oxidative changes in the experimental model of scopolamine-induced memory dysfunction.

2. MATERIAL AND METHODS

The present study was approved by the Ethics and Animal Welfare Committee of Federal University of Santa Maria, RS, Brazil.

Animals

A total of 64 experimentally male Wistar rats weighing 200-250g were used. They were maintained at 25°C, on a 12h light/ 12h dark cycle, with water and food *ad libitum*. On the day of the experiment, animals were brought to the experimental room and allowed to

habituate to the environmental conditions for approximately 60 min before the beginning of the experiment.

Drugs

Hydrogen peroxide was obtained from Merck (Rio de Janeiro, Brazil). Tris (hydroxymethyl) aminomethane, 5,5'-dithiobis-(2-nitrobenzoic acid), scopolamine hydrobromide, thiobarbituric acid and reduced glutathione were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid, n-butyl alcohol and tween 80 were obtained from Vetec (São Paulo, Brazil).

The novel pyrazoline compound 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1H-1-(2-hydroxybenzoyl)-pyrazole (Fig. 1) was synthesized from cyclocondensation reaction of the hydrazide methyl carboxylate with 1,1,1-trifluoro-4-methoxy-3-penten-2-one according to previously reported procedures (Martins *et al*., 2006). Analysis of the ^{1}H and ^{13}C NMR and mass spectra showed analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of compound was determined by GC/MS and amounted to 98-100%.

Inhibitory avoidance test

The inhibitory avoidance test apparatus consisted of a 25 x 25 x 35 cm box with a grid floor whose left portion was covered by a 7 x 25 cm plataform 2.5 cm high.

Habituation session: one habituation session was conducted in which each animal was first gently placed in the plataform for 5 min and returned to its home cage. The animals were then gently placed in the plataform and the latency to step-down the plataform with all four feet was measured in seconds. Animals that stepped down the plataform in less than 4 s or more than 20 s were noted to be hyperactive or apathetic, respectively and, therefore, were excluded from the experiment. Such excluded animals were replaced by other ones. The

habituation session was performed on these animals to reach an equal number of animals (8) with latencies between 4 and 20 s in each group.

Training session: thirty minutes after the habituation session, rats were subjected to a single training session in the step-down inhibitory avoidance apparatus. The rat was placed gently on the platform facing the rear left corner, and when the rat stepped down with all four paws on the grid, an electrical shock (0.6 mA/1 s) was delivered to the grid only during this training session. The animal was then returned to its home cage and tested for retention 24h later.

Test session: twenty-four hours after training, each rat was introduced to the step-down inhibitory avoidance apparatus and the latency (s) for the rat to step down with all four paws on the grid was measured, indicating memory level. An upper cutoff time of 300 s was set and all tests were run between 10:00 and 15:00 h. After this retrieval test session, animals were decapitated and skulls were split on ice. The whole brain of each animal was separated, weighed and homogenized in 5 volumes of ice-cold 150 mM NaCl, 10 mM phosphate buffer, pH 7.4. The homogenate was centrifuged at 3000 *x g* at 4ºC for 10 minutes to yield a low-speed supernatant that was used to determine non-protein thiol groups, thiobarbituric acid reactive substances and antioxidant enzymes activities.

Experimental design and drug treatment

The pyrazole compound was suspended in 5% tween 80, while scopolamine hydrobromide was dissolved in 0.9% NaCl. Control animals received respective vehicle injections, and they were run concurrently with drug-treated groups. Drugs and vehicles were administered intraperitoneally in a volume of 1 mL/kg body weight.

Animals that had a latency time between 4 and 20 s in the habituation session were randomly divided into eight experimental groups (8 rats per group). Sixty min before training

rats were injected with vehicle or pyrazole compound (100, 300 or 1000 μmol/kg) and thirty min later they were injected with saline or scopolamine (1.4 mg/kg). Scopolamine dose was chosen based on the study of El-Sherbiny *et al* (2003), while the dose of the pyrazole compound was based on studies that demonstrated a pharmacological antinociceptive activity for other pyrazole compounds (Tabarelli *et al*., 2004).

Non-protein thiol groups

One volume of the low-speed supernatant fraction was mixed with 1 volume of 10% trichloroacetic acid, followed by centrifugation and neutralization of the supernatant (to pH 7.5) with 1 M Tris as described by Jacques-Silva et al. (Jacques-Silva *et al*., 2001). Non-protein thiol groups were immediately determined as described by Ellman (Elman, 1959) at 412 nm after reaction with 5,5'-dithio-bis-(2-nitrobenzoic acid). A standard curve of cysteine was used to calculate the content of non-protein thiol groups in brain tissue samples.

Lipid peroxidation.

The supernatant was used for determination of thiobarbituric acid reactive species as described by Ohkawa et al. (Ohkawa *et al*., 1979). Following incubation samples were extracted with *n*-butanol and the reaction product was determined at 535 nm using a standard curve of 1,1,3,3-tetraethoxypropane.

Antioxidant enzymes

Superoxide dismutase activity was determined spectrophotometrically based on its ability to inhibit the autooxidation of epinephrine to adrenochrome at alkaline pH, according by McCord and Fridovich (McCord & Fridovich, 1969). One unit of SOD is the amount of enzyme that inhibits the oxidation of adrenaline by 50%.

Catalase activity was measured spectrophotometrically, as described by Aebi (Aebi, 1984) using hydrogen peroxide as substrate. The pseudo-first order reaction constant (*k*) of the decrease in H_2O_2 absorption at 25ºC was determined and enzyme specific activity was expressed as *k*/g protein.

Protein quantification

Protein was measured according to Lowry et al. (Lowry *et al*., 1951) using bovine serum albumin as the standard.

Statistical analysis

Data on the biochemical assays were analyzed using two-way analysis of variance (4 pyrazole doses x 2 scopolamine). Behavioural data was analysed by Kruskall-Wallis *H* test analysis of variance followed by multiple comparison Nemenyi nonparametric test. Results were considered statistically significant when $P<0.05$.

3. RESULTS

Kruskal-Wallis *H* test revealed a significant effect of treatment on the performance of rats in the inhibitory avoidance test ($H=28.72$, df=7, $P<0.05$; Fig. 2). Post hoc analysis revealed that scopolamine administration 30 min before training session resulted in shorter latency to step-down during the test session as compared to control groups ($P<0.05$). Pyrazole compound had no effect *per se* on the step-down latency. Results revealed a bell-shaped curve for the antiamnesic effect of pyrazole compound in the scopolamine model. Pre-treatment with 100 µmol/kg pyrazole compound completely prevented the reduction of step-down latency caused by scopolamine, while 300 µmol/kg partially prevented and 1000 µmol/kg had no effect (Fig. 2).

Two-away ANOVA did not show a significant effect of scopolamine or pyrazole compound on brain non-protein thiol groups levels, thiobarbituric acid reactive substances, SOD or CAT activities (Table 1).

4. DISCUSSION

Postmortem studies of the brains from AD patients showed increased levels of markers of oxidative damage, which include changes in antioxidant enzymes (Leutner *et al*., 2000; Wong *et al*., 2001), advanced glycation end products (Behl *et al*., 1994), lipid peroxidation (Hensley *et al*., 1994; Mark *et al*., 1997), free carbonyls (Smith *et al*., 1996; Smith *et al*., 1997), and peroxynitration (Good *et al*., 1996; Morris *et al*., 1998). These findings suggest that antioxidants could be useful in the treatment of AD. Reinforcing this proposal several recent studies revealed beneficial effects of diets supplemented with vitamins C and E for AD patients (Morris *et al*., 2002; Zandi *et al*., 2004; Grundman *et al*., 2004; Lange *et al*., 2007). Therefore, the interest for evaluating the efficacy of other antioxidants in AD models has increased.

In this study, we evaluated the effect of pretreatment with 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1H-1-(2-hydroxybenzoyl)-pyrazole against scopolamine-induced amnesia. This compound was recently demonstrated to prevent brain lipid peroxidation and H_2O_2-induced GSH oxidation (Martins et al., 2008). In agreement with previous studies (El-Sherbiny *et al*., 2003; Fan *et al*., 2004; Hung *et al*., 2004) systemic administration of scopolamine reduced memory performance in the inhibitory avoidance test. In addition, we found that systemic administration of 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1H-1-(2-hydroxybenzoyl)-pyrazole (100 μmol/kg) completely prevented the memory impairment induced by scopolamine.

Memory impairment associated with scopolamine treatment was shown to be linked to a decrease in central cholinergic neuronal activity following the blockade of the muscarinic receptors (Schon *et al*., 2005). However, a variety of compounds were demonstrated to be effective to protect against scopolamine-induced amnesia, indicating that mechanisms other than cholinergic system are also involved in this model. These compounds include cholinesterase inhibitors (Scipione et al., 2008), nitric oxide donors (Pitsikas *et al*., 2001) cannabinoid CB1 receptor antagonists (Takahashi *et al*., 2005) and compounds with antioxidant (Kumar et al., 2000; Fan *et al*., 2005); and anti-inflammatory activity (Howes *et al*., 2003). Currently acetylcholinesterase inhibitors are the only drugs approved for the treatment of cognitive dysfunction in AD (Giacobin, 2001), but various drugs effective in the scopolamine-induced amnesia model, like antiinflammatory agents and antioxidants are now moving into clinical trials (Cutler & Sramek, 2001; Doraiswamy, 2002).

Although the impaired cognitive function in the scopolamine model has been recently suggested to be associated to elevated brain oxidative status, there is some disagreement on the changes in oxidative stress markers observed in this model (El-Sherbiny *et al*., 2003; Khalifa, 2004; Fan *et al*., 2005). Some authors reported increased MDA levels along with decreased GSH levels, and no change in SOD in the brain after scopolamine administration (El-Sherbiny *et al*., 2003; Khalifa, 2004). In contrast, Fan *et al.* (2005) found no change in MDA levels, but decreased SOD activity. In the present study the amnesic effect of scopolamine was not accompanied by changes in oxidative stress markers, including lipid peroxidation, GSH levels, SOD or CAT activity. Also, the pyrazole compound caused no change in the oxidative stress markers evaluated. These findings suggest that mechanisms other than oxidative stress underlie the amnesic effect of scopolamine and the protective effect of 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1H-1-(2-hydroxybenzoyl)-

pyrazole. However, considering the role of oxidative stress in AD, the previously reported antioxidant activity of this compound could have an additional benefit for AD patients.

Pyrazole-derived compounds are synthetic molecules with various pharmacologically relevant activities (Borne, 1995). The pharmacological activities described for some pyrazole derivatives seems to be of special interest for the treatment of AD. It was recently demonstrated that some compounds of a novel series of 1-thiocarbamoyl-3-substituted phenyl-5-(2-pyrrolyl)-4,5-dihydro-(1H)-pyrazole derivatives exhibits both anti-inflammatory and monoamine oxidase B inhibitory activities, and for this reason were suggested as potential drugs for the treatment of AD (Gökhan-Kelekçi *et al*., 2007). Some 1-*N*-substituted thiocarbamoyl-3-phenyl-5-thienyl-2-pyrazoline derivatives have both cholinesterase and monoamine oxidase B inhibitory activity, which makes these drugs promising for the treatment of AD (Ucar *et al*., 2005). In addition, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamidehydrochloride) known as SR141716A and other pyrazole derivatives, including 4,5-dihydro-1H pyrazole derivatives different from the compound evaluated in the present study are antagonists of the CB1 cannabinoid receptor (Wolff *et al*., 2003; Cuberes-Altisen, 2007; Lange *et al*., 2007). These drugs were demonstrated to improve memory in inhibitory avoidance task, Morris Water Maze task and/or object recognition task and were patented as medicaments for the prophylaxis and/or treatment of one or more types of dementia selected from the group consisting of memory loss, vascular dementia, mild cognitive impairment and front temporal dementia (Cuberes-Altisen, 2007; Lange *et al*., 2007). AD accounts for most cases of dementia that are diagnosed after the age of 60 years in life (Brookmeyer *et al*., 1998). Therefore, the protective effect of 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-1-(2-hydroxybenzoyl)-pyrazole against scopolamine-induced amnesia could be related to some of these pharmacological activities previously reported for other pyrazole derivatives.

In conclusion, 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1*H*-1-(2-hydroxybenzoyl)-pyrazole seems to be a promising drug for the treatment of dementia. Further studies should be directed at evaluating the mechanism of action of this compound, as well as its effect on other models of AD and on markers of oxidative stress in specific brain structures involved in learning and memory processes, like hippocampus and cortex.

ACKNOWLEDGEMENTS

Work supported by PRONEX-FAPERGS-CNPq (grant 0408660 to M.A.P. Martins). D.M. Martins is the recipient of CAPES Master degree Fellowship and T.E. is the recipient of CNPq research Fellowship (proc. 306432/2007-2). S. Somacal is the recipient of a FAPERGS scientific initiation Fellowship.

REFERENCES

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

Behl C, Davis JB, Lesley R et al (1994) Hydrogen peroxide mediates amyloid beta protein toxicity. Cell 77:817-827

Brookmeyer R, Gray S et al (1998) Projections of Alzheimer's disease in the United States and the public health impact of delaying disease onset. Am J Public Health 88:1337-1342

Butterfield DA, Drake J, Pocernich C (2001) Evidence of oxidative damage in Alzheimer's disease brain: central role for amyloid beta-peptide. Trends Mol Med 7:548-554

Cross CE, Halliwell B, Borish ET et al (1987) Oxygen radicals and human disease. Ann. Intern. Med. 107:526-545

Cruz R, Almaguer Melian W, Bergado Rosado JA (2003) Glutathione in cognitive function and neurodegeneration. Rev Neurol 36:877-886

Cuberes-Altisen MR (2007) Substituted pyrazoline compounds, their preparation and use as medicaments. World Intelectual Property Organization. Patent WO/2007/009686.

Cutler NR, Sramek JJ (2001) Review of the next generation of Alzheimer's disease therapeutics: challenges for drug development. Prog Neuro-psychoph 25:27-57

Doraiswamy PM (2002) Non-Cholinergic strategies for treating and preventing Alzheimer's disease. CNS Drugs 16:811-824

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

El-Sherbiny DA, Khalifa AE, Attia AS et al (2003) Hypericum perforatum extract demonstrates antioxidant properties against elevated rat brain oxidative status induced by amnestic dose of scopolamine. Pharmacology, Biochemistry and Behavior 76:525-533

Fan Y, Hu J, Li J et al (2005) Effect of oligosaccharide sugar chain on scopolamine-induced memory impairment in rats and its related mechanisms. Neurosc Lett 374:222-226

Frussa-Filho R, Rocha JBT, Conceicão IM et al (1996) Effects of dopaminergic agents on visceral pain measured by the mouse writhing test. Arch Int Pharmacodyn Ther 33: 74-93.

Giacobin E (2001) Selective inhibitors of butyrylcholinesterase: a valid alternative for therapy of Alzheimer's disease? Drugs Aging 18:891-898

Godoy MCM, Fighera MR, Souza FR et al (2004) α_2-Adrenoceptors and 5-HT receptors mediate the antinociceptive effect of new pyrazolines, but not of dipyrone. Eur J Pharmacol 496: 93-97

Good P et al (1996) Evidence of neuronal oxidative damage in Alzheimer's disease. Am J Pathol 149:21-28

Good PF, Werner P, Hsu A et al (1996) Evidence of neuronal oxidative damage in Alzheimer's disease. Am J Pathol 149:21-28

Grundman M, Petersen RC, Ferris SH et al (2004) Mild cognitive impairment can be distinguished from Alzheimer disease and normal aging for clinical trials. Arch Neurol

61:59-66

Hensley K, Carney JM, Mattson MP et al (1994) A model for beta-amyloid aggregation and neurotoxicity based on free radical generation by the peptide: relevance to Alzheimer disease. Proc Natl Acad Sci 91:3270-3274

Howes MJR, Houghton PJ (2003) Plants used in Chinese and Indian traditional medicine for improvement of memory and cognitive function. Pharmacol Biochem Behav 75:513-527

Hung C, Lin M, Liao J et al (2004) Scopolamine-induced amnesia can be prevented by heat shock pretreatment in rats. Nerosci Lett 364:63-66

Illic TV, Jovanovic M, Jovicic A et al (1999) Oxidative stress indicators are evaluated in de novo Parkinson's disease patients. Funct Neurol 19:141-147

Jaques-Silva MC, Nogueira CW, Broch LC et al (2001) Diphenyl diselenide and ascorbic acid changes deposition of selenium and ascorbic acid in liver and brain of mice. Pharmacol Toxicol 88:119-125

Kang SY, Lee KY, Park MJ et al (2003) Decursin from Angelica gigas mitigates amnesia induced by scopolamine in mice. Neurobiology of Learning and Memory 79:11-18

Kumar V, Singh PN, Muruganandam AV et al (2000) Effect of Indian Hypericum perforatum Linn on animal models of cognitive dysfunction. J Etnopharmacol 72:119-128

Lange JHM, Iwema B, Wouter I, Van Vliet, Bernard J, Van Der Neut, Martina A.W. (2007) 4,5-Dihydro-(1H)-pyrazole derivatives as cannabinoid CB1 receptor modulators. United States Patent 20070142362.

Leutner S, Czech C, Schindowski, K et al (2000) Reduced antioxidant enzyme activity in brains of mice transgenic for human presenilin-1 with single or multiple mutations. Neurosc Lett 292:87-90

Lowry DH, Rosebrough NJ, Farr AL et al (1951) Protein measurement with the folinphenol reagent. J Biol Chem 193:265-275

Marcus DL, Thomas C, Rodriguez C et al (1998) Increased peroxidation and reduced antioxidant enzyme activity in Alzheimer's disease. Exp Neurol 150:40-44

Mark RJ, Pang Z, Geddes JW et al (1997) Amyloid beta-peptide impairs glucose transport in hippocampal and cortical neurons: involvement of membrane lipid peroxidation. J Nerosci 17:1046-1054

Martins MAP, Beck P, Machado P et al (2006) Microwave-assisted of novel 5-trichloromethyl-4,5-dihydro-1*H*-1-pyrazole methyl esters under solvent free conditions. J. Braz. Chem. Soc. 17:408-411

Matés JM, Pérez-Gómez C, Castro IN (1999) Antioxidant enzymes and human diseases. Clin Biochem 32:595-603

McCord JM, Fridovich I (1969) Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem 244:6049-6055

Mecocci P et al. (1997) Mitochondrial membrane fluidity and oxidative damage to mitochondrial DNA in aged and AD human brain. Mol Chem Neuropathol 31:53-64

Mecocci P, MacGarvey U, Beal MF (1994) Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease. Ann Neurol 36:747-751

Mello CF, Begnini J, De La Vega DD et al (1996) Antinociceptive effects of purine nucleotides. Braz J Med Biol Res 29: 1-9

Morris MC, Beckett LA, Scherr PA et al (1998) Vitamin E and vitamin C supplement use and risk of incident Alzheimer disease. Alzheimer Dis Assoc Disord 12:121-126

Morris MC, Evans DA, Bienias JL (2002) Dietary intake of antioxidant nutrients and the risk of incident Alzheimer disease in a biracial community study. JAMA 287:3230-3237

Nunomura G et al (1999) RNA oxidation is a prominent feature of vulnerable neurons in Alzheimer's disease. J Neurosci 19:1959-1964

Nunomura G et al (2001) Oxidative damage is the earliest event in Alzheimer disease. J Neuropathol Exp Neurol 60:759-767

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

Palmer AM (1999) The activity of the pentose phosphate pathway in increased in response to oxidative stress in Alzheimer's disease. J Neural Transm 106:317-328

Pappolla MA, Chyan YJ, Omar RA et al (1998) Evidence of oxidative stress and in vivo neurotoxicity of beta-amyloid in a transgenic mouse model of Alzheirmer's disease: a chronic oxidative paradigm for testing antioxidant therapies in vivo. Am J Pathol 152:871-877

Pitsikas N, Philippu A (1992) Different effects of tropisetron and ondansetron in learning and memory paradigms. Pharmacol Biochem Behav 56:571-576

Pitsikas N, Rigamonti AE, Cella SG et al (2001) Effects of molsidomine on scopolamine-induced amnesia and hypermoltility in the rat. Eur J Pharmacol. 426:193-200

Prokopp CR, Rubin MA, Sauzem PD (2006) A pyrazolyl-thiazole derivative causes antinociception in mice. Braz Journal of Med and Biol Res 39:795-799

Sayre LM et al (1997) 4-Hydroxynonenal-derived advanced lipid peroxidation end products are increased in Alzheimer's disease. J Neurochem 68:2092-2097

Schon K, Atri A, Hasselmo ME et al (2005) Scopolamine reduces persistent activity related to long- term encoding in the parahippocampal gyrus during delayed matching in humans. J Neurosci. 25:9112-9123

Scipione L, De Vita D, Musella A et al (2008) 4-Aminopyridine derivatives with anticholinesterase and antiamnesic acitivity. Bioorg Med Chem Lett. 18:309-312

Sivaprasad R, Nagaraj M, Varalakshmi P (2004) Combined efficacies of lipoic acid and 2,3-dimercaptosuccinic acid against lead-induced lipid peroxidation in rat liver. J Nutr

Biochem 15:18-23

Smith et al (1997) Widespread peroxynitrite-mediated damage in Alzheimer's disease. J Neurosci 17:2653-2657

Smith MA et al (1996) Oxidative damage in Alzheimer's. Nature 382:120-121

Smith MA, Perry G, Richey, PL et al (1996) Oxidative damage in Alzheimer's. Nature 382:120-121

Smith MA, Porteous CM, Coulter CV et al (1997) Selective targeting of an antioxidant to mitochondria. Eur J Biochem 263:709-716

Smith MA, Sayre LM, Monnier VM et al (1995) Radical AGEing in Alzheimer's disease. Trends Neurosci 18:172-176

Souza FR, Souza VT, Ratzlaff V et al (2002) Hypothermic and antipyretic effects of 3-methyl- and 3-phenyl-5-hydroxy-5-trichloromethyl-4,5-dihydro-1*H*-pyrazole-1-carboxyamides in mice. Eur J Pharmacol 451:141-147

Takahashi RN, Pamplona FA, Fernandes MS et al (2005) The cannabinoid antagonist SR141716A facilitates memory acquisition and consolidation in the mouse elevated T-maze. Neurosci Lett 380:270-275

Tomazetti J, Ávila DS, Ferreira APO et al (2004) Baker yeast-induced fever in young rats: Characterization and validation of an animal model for antipyretics screening. J Neurosci Meth 37 :1531-1540

Tsai A, Palmer G, Xiao G et al (1998) Structural characterization of arachidonyl radicals formed by prostaglandin H synthase-2 and prostaglandin H-synthase-1 reconstituted with mangano protoporphyrin IX. J Biol Chem 273:3888-3894

Ucar G et al (2005) 1-*N*-Substituted thiocarbamoyl-3-phenyl-5-thienyl-2-pyrazolines: A novel cholinesterase and selective monoamine oxidase B inhibitors for the treatment of Parkinson's and Alzheimer's diseases. Neurosci Lett 382:327-331

Van der Zee EA, Luiten PG (1999) Muscarinic acetylcholine receptors in the hippocampus, neocortex and amygdala: a review of immunocytochemical localization in relation to learning and memory. Prog Neurobiol 58: 409–471

Wolff MC, Leander JD (2003) SR 141716A, a cannabinoid CB1 receptor antagonist improves memory in a delayed radial maze task. Eur J Pharmacol 477:213-217

Wong A, Luth HJ, Deuther-Conrad W et al (2001) Advanced glycation endproducts co-localize with inducible nitric oxide synthase in Alzheimer's disease. Brain Res 920:32-40

Zandi PP, Anthony JC, Khachaturian AS et al (2004) Reduced risk of Alzheimer disease in users of antioxidant vitamin supplements: the Cache Country Study. Arch Neurol 61:82-88

Figure legends

Figure 1: Chemical structure of the novel pyrazoline derivative 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1H-1-(2-hydroxybenzoyl)-pyrazole.

Figure 2: Effect of pretreatment with 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1H-1-(2-hydroxybenzoyl)-pyrazole (0-1000 μmol/kg) and scopolamine (1.4 mg/kg) on the retrieval memory of an inhibitory avoidance task in adult rats. Performance was measured as the test step-down latency. *Significantly different from control groups and from SCO-100 μmol/kg pyrazole compound ($P<0.05$). Data are the median ± interquartile range for 8 animals in each group.

Table 1: Effect of pretreatment with 5-hydroxy-3-methyl-5-trifluoromethyl-4,5-dihydro-1H-1-(2-hydroxybenzoyl)-pyrazole and scopolamine (1.4 mg/kg) on the non-protein thiol groups levels (NPSH), TBARS level, SOD and CAT activity in rat brain

	Pyrazole compound (μmol/kg)	NPSH	TBARS	SOD	CAT
Control	0	0.032±0.005	0.45±0.03	8.63±0.77	4.47±0.97
	100	0.032±0.005	0.49±0.02	8.53±1.15	4.65±0.30
	300	0.035±0.005	0.49±0.02	7.92±0.91	5.24±1.03
	1000	0.030±0.005	0.45±0.02	7.00±0.75	3.95±0.57
SCO	0	0.041±0.006	0.53±0.08	7.86±1.22	3.82±0.99
	100	0.039±0.007	0.54±0.03	7.95±0.90	4.74±1.00
	300	0.037±0.008	0.52±0.04	8.17±1.50	4.17±0.41
	1000	0.040±0.007	0.47±0.04	7.52±0.96	4.46±1.01

Values are the mean±S.E, n=8. Data were analyzed by two-way ANOVA (4 pyrazole doses x 2 scopolamine doses).

Figure 1

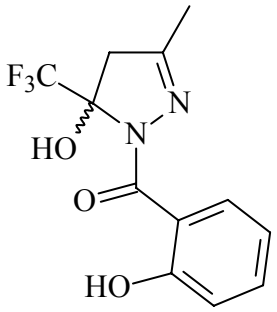
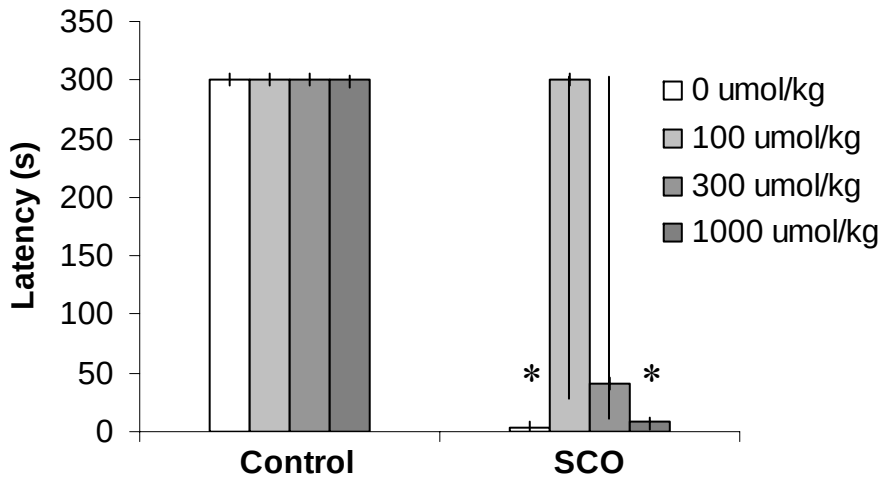


Figure 2


350
300
250
200
150
100
50
0
Latency (s)
Control
SCO
*
*
0 umol/kg
100 umol/kg
300 umol/kg
1000 umol/kg

4 DISCUSSÃO

As espécies reativas quando em excesso no ambiente celular podem causar estresse oxidativo, podendo levar a danos em vários componentes celulares, provocando perda da função dos mesmos. Assim, o estresse oxidativo tem sido associado a muitas doenças neurodegenerativas, incluindo a doença de Parkinson, a doença de Alzheimer e a esclerose lateral amiotrófica, entre outras (Halliwell & Gutteridge, 1999).

Considerando a necessidade de se desenvolver novos fármacos com capacidade de retardar e/ou evitar doenças neurodegenerativas, neste estudo, investigou-se o possível efeito antioxidante de derivados pirazolínicos (5-trifluorometil-4,5-diidro-1*H*-pirazol) *in vitro*. Foi também investigado o possível efeito antioxidante de um dos derivados pirazolínicos (5-hidroxi-3-metil-5-trifluorometil-4,5-diidro-1*H*-1-(2-hidroxibenzoil)-pirazol) em um modelo de déficit de memória induzido por escopolamina.

Os resultados do presente estudo indicam que dentre os seis compostos testados no ensaio *in vitro*, o composto 5 (5-hidroxi-3-metil-5-trifluorometil-4,5-diidro-1*H*-1-(2-hidroxibenzoil)-pirazol) foi o mais efetivo, pois apresentou um efeito pronunciado na redução da lipoperoxidação (redução dos níveis de TBARS) basal e induzida pelos pró-oxidantes (ferro, peróxido de hidrogênio e nitroprussiato de sódio) utilizados no ensaio, tendo efeitos significativos a partir de 15 µM quando comparado às mesmas condições na ausência do composto. Este efeito pode ser atribuído aos radicais presentes na estrutura do composto, já que o mesmo possui um anel aromático e também dois radicais hidroxil como substituintes na sua estrutura. Isso está em concordância com estudos que demonstram que estes tipos de substituintes conferem ao composto um caráter de removedor de radicais livres (Verhagen *et al*., 1996; Kefalas *et al*., 2003). Este foi o único composto dentre os testados que apresentou efeito na proteção da oxidação da GSH frente ao peróxido de hidrogênio, tendo efeito significativo na concentração de 150 µM. O efeito do composto neste caso não se deve a remoção direta do peróxido de hidrogênio. Portanto, supõe-se que o composto em questão atue em outro ponto da reação de oxidação, provocando o retardo e/ou bloqueio da mesma. Este composto também foi o que

teve maior atividade antioxidante total, demonstrada pela sua capacidade de remover o radical DPPH.

Os compostos 1 e 4 também reduziram a lipoperoxidação basal e induzida por ferro e nitroprussiato de sódio (SNP), tendo efeitos significativos a partir de 15µM quando comparado às mesma condições na ausência do composto. No entanto, a potência desses compostos foi inferior a do composto 5. A atividade antioxidante dos compostos 1 e 4 pode ser atribuída a estrutura básica desta série de compostos pirazolínicos.

Entre os compostos avaliados, o composto 2 teve o maior potencial antioxidante de redução do ferro (FRAP), seguido do composto 6, sugerindo que estes podem ser agentes redutores. No entanto, este poder redutor não foi confirmado no ensaio de DPPH que também avalia a capacidade dos compostos em transferir um único elétron. Além disso, estes compostos não apresentaram atividade antioxidante frente a peroxidação lipídica ou contra a oxidação da glutationa. Por outro lado, o composto 5, que foi o mais eficaz para evitar tanto a peroxidação lipídica quanto a oxidação da glutationa, estava entre os três compostos com menor atividade FRAP. Estes resultados indicam que o ensaio de FRAP não teve nenhuma correlação com a atividade antioxidante evidenciada nos outros modelos investigados.

O cérebro de pacientes com doença de Alzheimer (DA) apresenta aumento de marcadores de danos oxidativos, incluindo mudanças nas enzimas antioxidantes (Pappolla *et al*., 1998; Leuthner., 2000), peroxidação lipídica (Behl *et al*., 1994; Mark *et al*., 1997), carbonilação (Hensley *et al*., 1994; Smith *et al*., 1996), e peroxinitração de proteínas (Smith *et al*., 1996; Good *et al*., 1996). Assim, é possível que antioxidantes possuam um papel protetor contra as alterações neurológicas características da doença de Alzheimer. De fato, alguns ensaios clínicos com antioxidantes em pacientes com Alzheimer tiveram resultados promissores e tem impulsionado a procura por novas e mais eficazes terapias antioxidantes (Moreira *et al*., 2005). Outro estudo demonstrou que o tratamento com antioxidantes poderia evitar a propagação do dano tecidual e melhorar a sobrevivência neuronal. Também tem sido avaliado se a ingestão de antioxidantes, principalmente vitaminas, pode prevenir ou reduzir a progressão da doença de Alzheimer (Gilgun-Sherki *et al*., 2003).

Algumas evidências em estudos em animais e humanos indicam que antagonistas muscarínicos prejudicam a memória e função cognitiva no sistema nervoso central. A escopolamina, um antagonista muscarínico injetado aguda e sistemicamente, imita o déficit de memória observado na doença de Alzheimer, constituindo assim um modelo farmacológico útil para o estudo desta doença (Fan *et al*., 2005).

Para avaliar o possível efeito antioxidante do composto 5 *in vivo* no SNC, foi verificado o efeito do pré-tratamento com o mesmo em um modelo de déficit de memória usando escopolamina como indutor de amnésia.

A administração da escopolamina 30 min antes do teste de esquiva inibitória reduziu o tempo de latência dos animais em comparação ao controle. Isto está em concordância com alguns estudos que demonstram que a escopolamina causa amnésia em ratos após injeção intraperitoneal (El-Sherbiny *et al*., 2003; Hung *et al*., 2004; Fan *et al*., 2005). Nossos resultados indicam que o composto 5 na dose de 100 µmol/kg ip preveniu a amnésia induzida por escopolamina, o que está em concordância com patentes recentes de compostos pirazolínicos (distintos dos avaliados no presente estudo) para uso como medicamento para a profilaxia e/ou tratamento de demência por perda de memória, demência vascular, transtorno cognitivo leve e demência frontal temporal (Cuberes-Altisen, 2007; Lange *et al*., 2007). Outros estudos demonstraram que alguns compostos pirazolínicos podem agir como inibidores da monoamina oxidase B (MAO-B) e também de colinesterases, podendo ter características promissoras no tratamento de doenças de Alzheimer e Parkinson (Ucar *et al*., 2005).

Alguns estudos ressaltam a importância dos receptores canabinóides CB1 nos processos de aprendizagem e memória, sugerindo que os antagonistas desses receptores podem ser úteis no tratamento de distúrbios que envolvem déficits cognitivos (Wolff & Leander, 2003). Foi demonstrado que *N*-(piperidin-1-il)-5-(4-clorofenil)-1-(2,4-diclorofenil)-4-metil-1H-pirazol-3-carboxamidahidrocloreto), conhecido como SR141716A e outros derivados pirazolínicos, incluindo derivados 4,5-dihidro-1H pirazóis diferentes dos compostos avaliados no presente estudo, são antagonistas dos receptores canabinóides CB1 (Wolff & Leander, 2003; Lange *et al*., 2007). Estas drogas melhoraram a memória no teste da esquiva inibitória, do labirinto aquático de Morris e do reconhecimento de objetos e foram patenteados

como medicamentos para o tratamento da demência (Cuberes-Altisen, 2007; Lange *et al*., 2007).

Embora tenha sido recentemente sugerido que as alterações cognitivas no modelo da escopolamina estejam relacionadas à elevação do estado oxidativo cerebral, há algumas divergências sobre as alterações nos marcadores do estresse oxidativo observados neste modelo (El-Sherbiny *et al*., 2003; Khalifa, 2004; Fan *et al*., 2005). Alguns autores relataram aumento nos níveis de MDA juntamente com uma diminuição dos níveis de GSH, e nenhuma mudança na atividade da SOD no cérebro após administração de escopolamina (El-Sherbiny *et al*., 2003; Khalifa, 2004). Em contraste, Fan et al. (2005) não encontraram nenhuma mudança nos níveis de MDA, mas relataram uma diminuição da atividade da SOD. No presente estudo, o efeito amnésico da escopolamina não foi acompanhado por mudanças nos marcadores do estresse oxidativo, incluindo peroxidação lipídica, níveis de GSH e atividade da SOD ou CAT. Além disso, o composto pirazolínico não causou qualquer alteração nos marcadores de estresse oxidativo avaliados. Estes resultados sugerem que outros mecanismos diferentes do estresse oxidativo estão envolvidos no efeito amnésico da escopolamina e no efeito protetor do 5-hidroxi-3-metil-5-trifluorometil-4,5-diidro-1H-1-(2-hidroxibenzoil)-pirazol. No entanto, considerando o papel do estresse oxidativo na doença de Alzheimer, a atividade antioxidante desse composto, observada *in vitro*, poderia ser um benefício adicional para pacientes com a doença de Alzheimer.

Os resultados obtidos no presente estudo indicam que o derivado pirazolínico 5 teve atividade antioxidante pronunciada *in vitro*, em comparação aos demais compostos testados. Na avaliação de seu efeito sobre os danos induzidos pela escopolamina, pode-se concluir, a partir dos resultados obtidos, que na dose de 100 µmol/kg, o composto reverteu a amnésia induzida por escopolamina. Estudos futuros deverão ser realizados para avaliar o mecanismo da ação anti-amnésica do composto 5, assim como a sua ação sobre os níveis de estresse oxidativo no hipocampo e no córtex cerebral separadamente, uma vez que estas estruturas estão envolvidas nos processos de aprendizagem e memória.

5 CONCLUSÕES

Os resultados do presente trabalho indicam que:

→ O composto (**5**) 5-hidroxi-3-metil-5-trifluorometil-4,5-diidro-1*H*-1-(2-hidroxibenzoil)-pirazol apresentou maior atividade antioxidante *in vitro* dentre os compostos avaliados, evidenciada pela sua capacidade de remover o radical DPPH, e na proteção da lipoperoxidação e da oxidação da GSH, o que provavelmente está relacionado ao substituinte hidroxibenzoil em sua estrutura;

→ Os compostos (**1**) 5-hidroxi-3-metil-5-trifluorometil-4,5-diidro-1*H*-carbaldeido-pirazol e (**4**) 5-hidroxi-3-metil-5-trifluorometil-4,5-diidro-1*H*-1-benzoil-pirazol também apresentaram atividade antioxidante contra a lipoperoxidação *in vitro*, mas menor quando comparados ao composto (**5**), sugerindo que a estrutura básica da série de derivados pirazolínicos possui atividade antioxidante;

→ A baixa ou nula atividade antioxidante dos compostos (**2**) 5-hidroxi-3-metil-5-trifluorometil-4,5-diidro-1*H*-1-acetil-pirazol, (**3**) 5-hidroxi-3-metil-5-trifluorometil-4,5-diidro-1*H*-carboxiamida-pirazol e (**6**) 5-hidroxi-3-metil-5-trifluorometil-4,5-diidro-1*H*-1-(4-metoxibenzoil)-pirazol nos ensaios *in vitro* sugere que a introdução de radicais metila, amina e metoxibenzoila na estrutura básica da série de compostos pirazolínicos avaliados reduz a atividade dos mesmos.

→ O composto (**5**) protegeu contra a amnésia induzida por escopolamina na tarefa da esquiva inibitória. No entanto, nem a escopolamina, nem este composto causaram alterações oxidativas no cérebro dos ratos, sugerindo que o efeito protetor do composto não está relacionado a sua atividade antioxidante.

→ O composto (**5**) parece ser promissor para o tratamento da demência. No entanto, outros estudos são necessários para elucidar os mecanismos envolvidos na ação anti-amnésica deste composto, bem como o seu efeito em outros modelos de demência, além da induzida por escopolamina.

6 REFERÊNCIAS BIBLIOGRÁFICAS

ADAM-VIZI, V. Production of reactive oxygen species in brain mitochondria: contribuition by electron transport chain and nonelectron transport chain sources. **Antioxidant and Redox Signaling.** v. 7, p. 1140-1149, 2005.

AGARWAL, V.; PATEL, S.; PANK, K.K. H_2 production by steam reforming of methanol over $Cu/ZnO/Al_2O_3$ catalysts:transient deactivation kinetics modeling. **Applied Catalysis A: General**. v. 279, p. 155-164, 2005.

AKBAS, E.; BERBER, I. Antibacterial and antifungal activities of new pyrazolo[3,4-*d*]pyridazin derivatives. **European Journal of Medicinal Chemistry**. v. 40, p. 401-405, 2005.

AKMAN, H. et al. A possible central antinociceptive effect of dypirone in mice. **Pharmacolgy.** v. 53, p. 71-78, 1996.

ANCELIN, M-L. et al. Is Antioxidant Therapy a Viable Alternative for Mild Cognitive Impairment? Examination of the Evidence. **Dementia and Geriatric Cognitive Disorders.** v. 24, p. 1-19, 2007.

ANDERSEN, J. K. Oxidative stress in neurodegeneration. Cause or consequence? **Nature Reviews Neuroscience.** v. 5, p. S18-S25, 2004.

ARGENTIERI, D. C. et al. Tepoxalin: A dual cyclooxygenase/5-lipoxygenase inhibitor of arachidonic acid metabolism with potent anti-inflammatory activity and a favorable gastrointestinal profile. **Journal of Pharmacology and Experimental Therapeutics.** v. 271, p.1127-1144, 1994.

BARREIROS, A. L. B. S.; DAVID, J. M. Estresse oxidativo: relação entre espécies reativas e defesa do organismo. **Química Nova.** v. 29, p. 113-123, 2006.

BASSET, C. N.; MONTINE, T. J. Lipoproteins and lipid peroxidation in Alzheimer's disease. **Journal of Nutrition, Health and Aging.** v. 7, p. 446-451, 2003.

BASTIANETTO, S.; ZHENG, W. H.; QUIRION, R. Neuroprotective abilities of resveratrol and other red wine constituints against nitric oxide-related toxicity in cultured hippocampal neurons. **Brazilian Journal of Pharmacology**. v. 131, p. 711-720, 2000.

BEHL, C. et al. Vitamin E protects nerve cells form amyloid β protein toxicity. **Biochemistry, Biohysical Research.** v. 186, p. 944-950, 1992.

BEHL, C. et al. Hydrogen peroxide mediates amyloid β protein toxicity. **Cell**. v. 77, p. 817-827, 1994.

BORNE, R. F. Nonsteroidal anti-inflamatory drugs. In: Foye, W. O; Lemke, T. L.; Williams, D. A. **Medicinal Chemistry**. Baltimore: Williams & Wilkins, 1995.

BRIGELIUS-FLOHE, R. Tissue-specific functions of individual glutathione peroxidases. **Free Radical Biology & Medicine.** v. 27, p. 951-965, 1999.

BUTTERFIELD, A. D. et al. Evidence of oxidative in Alzheimer's disease brain: central role of amyloid β-peptide. **Trends in Molecular Medicine**. v. 12, p. 548-554, 2001.

CANIATO, R. et al. Melatonin in plants. **Advanced Experimental Medicinal Biology**. v. 527, p. 593-597, 2003.

CASTEGNA, A. et al. Proteomic identification of nitrated proteins in Alzheimer's disease brain. **Journal of Neurochemistry.** v. 85, p. 1394-1401, 2003.

COSTA, D. et al. Inhibition of human neutrophil oxidative burst by pyrazoline derivatives. **Free Radical Biology & Medicine.** v. 40, p. 632-640, 2006.

COYLE, J. T.; PUTFARCKEN, P. Oxidative stress, glutamate and neurodegenerative disordens. **Science.** v. 262, p. 689-695, 1993.

CRUZ, R. et al. Glutathione in cognitive function and neurodegeneration. **Rev Neurol.** v. 36, p. 877-886, 2003.

CUBERES-ALTISEM, M. R. (2007). Substituted pyrazoline compounds, their preparation and use as medicaments. World Intelectual Property Organization 009686.

CUNICO, W. et al. Antimalarial activity of 4-(5-trifluorometyl-1*H*-pyrazol-1-yl)-chloroquine analogues. **Bioorganic & Medicinal Chemistry Letters.** v. 16, p. 649-653, 2006.

CUTLER, N. R.; SRAMEK, J. J. Review of the next generation of Alzheimer's disease therapeutics: challenges for drug development. **Prog. Neuro-psychoph.** v. 25, p. 27-57, 2001.

DORAISWAMY, P. M. Non-Cholinergic strategies for treating and preventing Alzheimer's disease. **CNS Drugs.** v. 16, p. 811-824, 2002.

DRINGEN, R.; PAWLOSKI, P. G.; HIRRLINGER, J. Peroxide detoxification by brain cells. **Journal of Neuroscience Research.** v. 79, p. 157-165, 2005.

EL-SHERBINY, D. A. et al. Hypericum perforatum extract demonstrates antioxidant properties against elevated rat brain oxidative status induced by amnestic dose of scopolamine. **Pharmacology, Biochemistry and Behavior**. v. 76, p. 525-533, 2003.

FAHN, S.; COHEN, G. The oxidant stress hypothesis in Parkinson's disease. Evidence Supporting it. **Annals of Neurology.** v. 32, p. 804-812, 1992.

FAN, Y. et al. Effect of oligosaccharide sugar chain on scopolamine induced memory impairment in rats and its related mechanisms. **Neuroscience Letters**. v. 374, p. 222-226, 2005.

FRIDOVICH, I. The biology of oxygen radicals. **Science**. v. 201, p. 875-880, 1978.

FRUSSA-FILHO, R. et al. Effects of dopaminergic agents on visceral pain measured by the mouse writhing test. **Archives Internationales of Pharmacodynamie et de Therapie.** v. 331, p. 74-93, 1996.

FUNK, J. L.; MORTEL, K. F.; MEYER, J. S. Effects of estrogen replacement therapy on cerebral perfusion and cognition among postmenopausal women. **Dementia.** v. 2, p. 268-272, 1991.

GIACOBIN, E. Selective inhibitors of butyrylcholinesterase: a valid alternative for theraphy of Alzheimer's disease? **Drugs Aging**. v. 18, p. 891-898, 2001.

GILGUN-SHERKI, Y. et al. Antioxidant treatmente in Alzheimer's disease: current state. **Journal of Molecular Neuroscience.** v. 21, p. 1-11, 2003.

GODOY, M. C. M. et al. α_2-Adrenoceptors and 5-HT receptors mediate the antinociceptive effect of new pyrazolines, but not of dipyrone. **European Journal of Pharmacology**. v. 496, p. 93-97, 2004.

GOOD, P. F. Evidence for neuronal oxidative damage in Alzheimer's disease. **American Journal of Pathology**. v. 149, p. 21-28, 1996.

GUPTA, Y. K.; BRIYAL, S.; CHAUDHARY, G. Protective effect of *trans*-resveratrol against kainic acid-induced seizures and oxidative stress in rats. **Pharmacology Biochemistry and Behavior**. v. 71, p. 253-257, 2002.

GÜRSOY, A. et al. Synthesis and preliminary evaluation of new 5-pyrazolinone derivatives as analgesic agents. **European Journal of Medicinal Chemistry.** v. 35, p. 359-364, 2000.

HALLIWELL, B.; GUTTERIDGE, J. M. C. The Chemistry of free radicals and related 'reactive species' In: Free Radicals in biology and medicine. **Oxford University Press**, New York, p. 36-104, 1999.

HAN, Y. –S. et al. Neuroprotective effects of resveratrol against β-amyloid-induced neurotoxicity in rat hippocampal neurons: involvement of protein kinase C. **Brazilian Journal of Pharmacology.** v. 141, p. 997-1005, 2004.

HARDELAND, R. Antioxidative protection by melatonin – Multiplicity of mechanisms form radical detoxification to radical avoidance. **Endocrine**. v. 27, p. 119-130, 2005.

HARRIS, M. E. et al. Direct evidence of oxidative injury produced by the Alzheimer's β-amyloid peptide (1-40) in cultured hippocampal of neurons. **Exp Neurology.** v. 131, p. 193-202, 1995.

HERNANDEZ, N .; VANEGAS, H. Antinociception induced by PAG-microinjected dipyrone (metamizol) in rats: involvement of spinal endogenous opioids. **Brain Research.** v. 896, p. 175-178, 2001.

HENSLEY, K. et al. A model of β-amyloid aggregation and neurotoxicity based on free radical generation by the peptide: relevance to Alzheimer's disease. **Proc Natl Acad Sci USA.** v. 91, p. 3270-3274, 1994.

HERBETTE, S. et al. Seleno-independent glutathione peroxidases. More than simple antioxidant scavengers. **European Journal of Biochemistry.** v. 274, p. 2163-2180, 2007.

HOWES, M. J. R.; HOUGHTON, P. J. Plants used in Chinese and Indian traditional medicine for improvement of memory and cognitive function. **Pharmacology, Biochemistry and Behavior.** v. 75, p. 513-527, 2003.

HUNG, C.; LIN, M.; LIAO, J. Scopolamine-induced amnesia can be prevented by heat shock preatreatment in rats. **Neuroscience Letters.** v. 364, p. 63-66, 2004.

HUANG, S. S. et al. Resveratrol reduction of infarct size in Long-Evans rats subjected to focal cerebral ischemia. **Life Sciences**. v. 69, p. 1057-1065, 2001.

ISHII, P. G. et al. Transcription factor nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. **Journal of Biological Chemistry**. v. 272, p. 16023-16029, 2000.

IZQUIERDO, I. **Memória**. Editora Artmed S.A., Porto Alegre, 2002.

ISKANDER, K. et al. NQO1 and NQO2 regulation of humoral immunity and autoimmunity. **Journal of Biological Chemistry.** v. 281, p. 30917-30924, 2006.

INSEL, P. A. Analgesic – antipyretic and anti-inflammatory agents and drugs employed in the treatment of gout. **Pharmacological Basis of Therapeutics.** Atlampa: Mc Graw Hill Interamericana, 1996.

JAFFE, A. B. et al. Estrogen regulates metabolism of Alzheimer amyloid beta precursor protein. **Journal of Bilogy Chemistry.** v. 269, p. 13065-13068, 1994. p. 747-751, 1994.

JAISWAL, A. K. Regulation of genes encoding NAD(P)H:quinine oxidoreductases. **Free Radical Biology and Medicine.** v. 29, p. 254-262, 2000.

JEONG, T-S. et al. Novel 3,5-diaryl pyrazolines as low-density lipoprotein (LDL) oxidation inhibitor. **Bioorganic & Medicinal Chemistry Letters**. v. 14**,** p. 2719-2723, 2004.

JOHNSON, F.; GIULIVI, C. Superoxide dismutases and their impact upon human health. **Molecular Aspects of Medicine.** v. 26, p. 340-352, 2005.

KEFALAS, P. et al. A comparative study on the in vitro antiradical efficiency and hydroxyl free radical scavenging activity in aged red wines. **Food Science and Tecnhology International.** v. 9, p. 383-387, 2003.

KIM, Y. et al. Human prx1 gene is a target of Nrf2 and is up-regulated by hypoxia/reoxygenation: implication to tumor biology. **Cancer Research.** v. 67, p. 546-554, 2007.

KUMAR, V. et al. Effect of Indian Hypericum perforatum Linn on animal models of cognitive dysfunction. **J Ethnopharmacol.** v. 72, p. 119-128, 2000.

LANGE, J. H. M. et al. (2007). 4,5-Dihydro-(1H)-pyrazole derivatives as cannabinoid CB1 receptor modulators. United States Patent 20070142362.

LECANNELIER, S. Antiinflamatorios no esteroideos. In: Marcondes, **J Farmacologia**. Buenos Aires: Intermédica, 1976.

LI, Y.; JAISWAL, A. K. Regulation of human NAD(P)H:quinome oxidoreductase gene. Role of AP1 binding site contained within human antioxidant response element. **Journal of Biological Chemistry.** v. 21, p. 15097-15104, 1992.

LORENZETTI, B. B.; FERREIRA, S. H. Mode of analgesic action of dipyrone: direct antagonism of inflamatory hyperalgesia. **European Journal of Pharmacology.** v. 114, p. 375-381, 1985.

MAGGIO, B. et al. Synthesis and pharmacological study of ethyl 1-methyl-5-(substituted 3,4-dihydro-4-oxoquinazolin-3-yl)-1H-pyrazole-4-acetates. **European Journal of Medicinal Chemistry.** v. 36, p. 737-742, 2001.

MAIER, C. M.; CHAN, P. H. Role of superoxide dismutases in oxidative damage and neurodegenerative disorders. **Neuroscientist**. v. 8, p. 323-334, 2002.

MARCUS, D. L. et al. Increased peroxidation are reduced antioxidant enzyme activity in Alzheimer's disease. **Experimental Neurology.** v. 150, p. 40-44, 1998.

MARKLUND, S. L. et al. Copper-and-zinc-containing superoxide dismutase, manganese-containing superoxide dismutase, catalase, and glutathione peroxidase in normal and neoplastic human cell lines and normal human tissues. **Cancer Research.** v. 42, p. 1955-1961, 1982.

MARQUEZ, J. O.; FERREIRA, S. H. Regional dipyrone nociceptor blockade: a pilot study. **Brazilian Journal of Medical and Biological Research.** v. 20, p. 441-444, 1987.

MARK, R. J. et al. Amyloid β-peptide impairs glucose transport in hippocampal and cortical neurons: involvement of membrane lipid peroxidation. **The Journal of Neuroscience.** v. 17, p. 1046-1054, 1997.

MARKLUND, S. L. Extracellular superoxide dismutase in human tissues and human cell lines. **The Journal of Clinical Investigation.** v. 74, p. 1398-1403, 1984.

McCORD, J. M.; EDEAS, M. A. SOD, oxidative stress and human pathologies: a brief history and a future vision. **Biomedicine & Pharmacotherapy.** v. 59, p. 139-142, 2005.

McCORD, J.M.; FRIDOVICH, I. Superoxide dismutase. An enxymic function for erythrocuprein (hemocuprein). **Journal of Bilogical Chemistry.** v. 244, p. 6049-6055, 1969.

McCORD, J. M. Iron-and manganese-containing superoxide dismutases:structure, distribution, and evolutionary relationships. **Advances in Experimental Medicine and Biology.** v. 74, p. 540-550, 1976.

MECOCCI, P.; MACGARVEY, U.; BEAL, M.F. Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease. **Annals of Neurology**. v. 36, p. 747-751, 1994.

MELLO, C. F. et al. Antinociceptive effects of purine nucleotides. **Brazilian Journal Medicinal Biological Research.** v. 29, p. 1-9, 1996.

MOLAVI, B.; MEHTA, J.L. Oxidative stress in cardiovascular disease: Molecular basis of its deleterious effects, its detection, and therapeutic considerations. **Current Opinion in Cardiology.** v. 19, p. 488-493, 2004.

MOREIRA, P. I. et al. Oxidative damage and Alzheimer's disease: are antioxidant therapies useful? **Drugs News & Perspectives.** v. 18, p. 13-19, 2005.

NORDBERG J.; ARNÉR, E.S.J. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. **Free Radical Biology & Medicine.** v. 31, p. 1287-1312, 2001.

OCHI, T. et al. Anti-inflamatory and analgesic effects of a novel pyrazole derivative, FR140423. **European Journal of Pharmacology.** v. 365, p. 259-266, 1999.

PAPPOLLA, M. A. et al. Evidence of oxidative stress and in vivo neurotoxicity of beta-amyloid in a transgenic mouse model of Alzheimer's disease: a chronic oxidative paradigm for testing antioxidant therapies in vivo. **American Journal of Pathology**. v. 152, p. 871-877, 1998.

PASTOR, N. et al. A detailed interpretation of OH radical footprints in a TBP-DNA complex reveals the role of dynamics in the mechanisms of sequence specific binding. **Journal of Molecular Biological.** v. 304, p. 55-68, 2000.

PITSIKSA, N. et al. Effects of molsidomine on scopolamine-induced amnesia and hypermotility in the rat. **Eur J Pharmacol.** v. 423, p. 193-200, 2001.

PRATICÒ, D. et al. Iron-dependent human platelet activation and hydroxyl radical formation: involvement of protein kinase C. **Circulation.** v. 99, p. 3118-3124, 1999.

PROKOPP, C. R. et al. A pyrazolyl-thiazole derivative causes antinoception in mice. **Brazilian Journal of Medicinal and Biological Research.** v. 39, p. 795-799, 2004.

RANATUNGE, R. R. et al. Synthesis and selective cyclooxygenase-2 inhibitory activity of a series of novel, nitric oxide donor containing pyrazoles. **Journal of Medicinal Chemistry.** v. 47, p. 2180-2193, 2004.

RANG, H. P.; DALE, M. M.; RITTER, J. M. **Pharmacology** 3ª ed. Edinburgh, London, Melbourne and New York:Churchill-Livingstone, 1995.

REITER, R. J. et al. Free radicals-mediated molecular damage. Mechanisms for the protective actions of melatonin in the central nervous system. **Annals of the New York Academy of Sciences.** v. 939, p. 200-215, 2001.

ROSLER, M. et al. Free radicals in Alzheimer's dementia: currently available therapeutic strategies. **Journal of neural transmission, Supplementum**. v. 54, p. 211-219, 1998.

RUBIN, M. A. et al. Anxiolytic-like Effects of 4-Phenyl-2-trichloromethyl-3H- 1,5-benzodiazepine Hydrogen Sulfate in Mice. **Brazilian Journal of Medicinal and Biological Research.** v. 33, p.1069-1073, 2000.

SANO, M. et al. A controlled trial of selegiline, α-tocopherol, or both as treatment for Alzheimer's disease. **The New England Journal of Medicine.** v. 336, p. 1216-1222, 1997.

SAVASKAN, E. et al. Red wine ingredient resveratrol protects from β-amyloid neurotoxicity. **Gerontology.** v. 49, p. 380-383, 2003.

SCHMELTZ, M.; WEBER, S.; KRESS, M. Topical acetyl salicylate and dipyrone attenuated neurogenic protein extravasation in rat skin in vitro. **Neuroscience Letters.** v. 290, p. 57-60, 2000.

SCHON, K et al. Scopolamine reduces persistent activity related to long-term encoding in the parahippocampal gyrus during delayed matching in humans. **J. Neurosci.** v. 25, p. 9112-9123.

SCIPIONE, L. et al. 4-Aminopyridine derivatives with anticholinesterase and antiamnesic activity. **Bioorg Med Chem Lett.** v. 18, p. 309-312, 2008.

SIVAPRASAD, R.; NAGARAJ, M., VARALAKSHMI, P. Combined efficacies of lipoic acid and 2,3-dimercaptosuccinic acid against lead-induced lipid peroxidation in rat liver. **Journal of Nutritional Biochemistry.** v. 15, p. 18-23, 2004.

SIES, H. What is oxidative stress? In: Kearney, J. F, Jr., ed. **Oxidative stress and vascular disease.** Boston: Kluwer Academic Publishers, p. 1-8, 2000.

SINHA, K.; CHAUDHARY, G.; GUPTA, Y. K. Protective effect of resveratrol against oxidative stress in middle cerebral artery occlusion model of stroke in rats. **Life Sciences.** v. 71, p. 655-665, 2002.

SMITH, M. A.; PERRY, G. Protein modification and interactions in Alzheimer's disease. In. **Alzheimer's Disease: Etiological Mechanisms and Therapeutic Possibilities.** eds J. D. Turner, K. Beyreuther and F. Theuring. Springer-Verlag, Berlin, p. 169-182, 1996.

SOUZA, F. R. et al. 3-Methyl-5-hydroxyl-5-tricholoromethyl-1H-1-pyrazolcarboxamide induces antinociception. **Pharmacology, Biochemistry and Behavior.** v. 68, p. 525-530, 2001.

SOUZA, F. R. et al. Hypothermic and antipyretic effects of 3-methyl- and 3-phenyl-5-hydroxy-5-trichloromethyl-4,5-dihydro-1*H*-pyrazole-1-carboxyamides in mice. **European Journal of Pharmacology.** v. 451, p. 141-147, 2002.

SUBBARAO, K. V.; RICHARDSON, J. S.; ANG, L. C. Autopsy samples of Alzheimer's cortex show increased peroxidation in vitro. **Journal of Neurochemistry.** v. 55, p. 342-345, 1990.

SULTANA, R. et al. Identification of nitrated proteins in Alzheimer's disease brain using a redox proteomics approach. **Neurobiology of Disease.** v. 22, p. 76-87, 2006b.

TABARELLI, Z. et al. Antinociceptive effect of novel pyrazolines in mice. **Brazilian Journal of Medical and Biological Research.** v. 37, p. 1531-1540, 2004.

TABARELLI, Z. et al. Antinociceptive effects of cremophor EL orally administered to mice. **Brazilian Jounal of Medicinal and Biological Research.** v. 36, p. 119-123, 2003.

TAIWO, Y. O.; LEVINE, J.D. Caracterization of the arachidonic acid metabolites mediating bradiking and noradrenaline hyperalgesia. **Brain Research.** v. 458, p. 402-406, 1988.

TAKAHASHI, R. N.; PAMPLONA, F. A.; FERNANDES, M. S. The cannabinoid antagonist SR141716A facilitates memory aquisition and consolidation in the mouse elevated T-maze. **Neuroscience Letters.** v. 380, p. 270-275, 2005.

THOMAS, T. Monoamine oxidase-B inhibitors in the treatment of Alzheimer's disease. **Neurobiology of aging.** v. 21, p. 343-348, 2000.

TOMAZETTI, J. et al. Baker yeast-induced fever in young rats: Characterization and validation of an animal model for antipyretics screening. **Journal of Neuroscience Methods**. v. 37, p. 1531-1540, 2004.

TSAI, A. et al. Structural characterization of arachidonyl radicals formed by prostaglandin H synthase-2 and prostaglandin H-synthase-1 reconstituted with mangano protoporphyrin IX. **Journal of Biology Chemistry.** v. 273, p. 3888-3894, 1998.

UCAR, G. et al. 1-*N*-Substituted thiocarbamoyl-3-phenyl-5-thienyl-2-pyrazolines: A novel cholinesterase and selective monoamine oxidase B inhibitors for the treatment

of Parkinson's and Alzheimer's diseases. **Neurosci Lett**. v. 382, p. 327-331, 2005.

VALKO, M. et al. Free radical and antioxidants in normal physiological functions and human disease. **The International Journal of Biochemistry & Cell Biology.** v. 39, p. 44-84, 2007.

VAN KAMPEN, J. et al. Neuroprotective actions of the ginseng extract G115 in two rodent models of Parkinson's disease. **Experimental Neurology.** v. 184, p. 21-29, 42003.

VARADAJAN, S. et al. Review: Alzheimer's amyloid beta-peptide-associated free radical oxidative stress and neurotoxicity. **Journal of Structural Biology.** v. 130, p. 184-208, 2000.

VERHAGEN, J. V.; HAENEN, G. R. M. M.; BAST, A. Nitric oxide radical scavenging by wines. **Journal of Agricultural and Food Chemistry**. v. 44, p. 3733-3734, 1996.

VILAÑO, D. et al. Radical scavenging ability of polyphenolic compounds towards DPPH free radical. **Talanta**. v. 71, p. 230-235, 2007.

ZARKOVIC, N. 4-Hydroxynonenal as a bioactive marker of pathophysiological processes. **Molecular aspects of Medicine.** v. 24, p. 281-291, 2003.

WAGENER, F. A. et al. Different faces of the heme-heme oxygenases system in inflammation. **Pharmacology Reviews.** v. 55, p. 551-571, 2003.

WANG, Q. et al. Resveratrol protects against global cerebral ischemic injury in gerbils. **Brain Research.** v. 958, p. 439-447, 2002.

WOLFF, M.C. & Leander, J.D. SR 141716A, a cannabinoid CB1 receptor antagonist improves memory in a delayed radial maze task. **Eur J Pharmacol**. V. 477, p. 213-217, 2003.

YAKSH, T. L. & HAMMOND, D. L. Peripheral and central substrates involved in the rostral transmissions of nociceptive information. **Pain.** v. 13, p. 1-85, 1982.

YAMAZAKI, T. et al. Effects of an aqueous extract of Puerariae flos (Thomsonide) on impairment of passive avoidance behavior in mice. **J. Ethnopharmacol.** v.100, p.244–248, 2005.

7 ANEXOS

7.1 ANEXO A – Roteiro para autores/Guia para a redação e edição de artigo científico a ser submetido à Revista Basic & Clinical Pharmacology & Toxicology

Organization of manuscripts

Manuscripts should be submitted in the English language (see above). They should be double-spaced and with a wide margin. Pages should be numbered consecutively, beginning with the title page, which should contain a concise title, institution(s) or laboratory(ies) where the work was done, names of all authors with first names spelled out, and an abbreviated form of the title (running title). Full mailing address in English for the corresponding author should also be given including telefax number and e-mail address.
Page 2 should contain an abstract of up to 250 words. The abstract should contain a summary of what was done, the results obtained, and valid conclusions drawn therefrom. The following pages should contain Introduction and background (long historical introductions should be avoided, a reference to bibliographies in handbooks or the like will suffice), followed by Materials and Methods, Results, Discussion which should incorporate the conclusion(s) drawn from the study, Acknowledgements, References, numbered tables with legends, and illustrations or graphs in high quality with legends on separate sheets.
Abbreviations in tables and figures should be explained in the legends. Text and footnotes must contain all the information necessary to understand and interpret the table without reference to the text.
Illustrations must be clear enough to permit readable reproduction. Symbols should be large enough to be readable also after reduction of the illustration.
The experimental results should on the whole be published only in the form of graph or tables, which must contain all the information necessary to understand the table or illustration without reference to the text.

Illustrations

Tables. Each table should have a brief, specific, descriptive title, giving sufficient explanation to make the data intelligible without reference to the text. Number all tables and cite in numerical order in the text, using Arabic numerals.

Preparation of figures. Cite figures in the text in numerical order using Arabic numerals. For peer-review submission, follow the online uploading instructions. Please save vector graphics (e.g. line artwork) in Encapsulated Postscript Format (EPS) and bitmap files (e.g. half-tones) in Tagged Image File Format (TIFF). Ideally, vector graphics that have been saved in metafile (.WMF) or pict (.PCT) format should be embedded within the body of the text file. Detailed information on our digital illustration standards is available at http://www.blackwellpublishing.com/authors/digill.asp. Always send a hard copy of digitally supplied figures to the Central Editorial Office (details above).

Figure sizing for accepted manuscripts. For the print publication, lay out figures as compactly as is consistent with conveying the relevant data. Figures will be sized to fit the smallest possible space, but in order to prevent radical changes in figure content, prepare the figures in one of two sizes: 8.0 cm (1-column width) or, if necessary, 11.5 cm (11/2 column width). These instructions do not apply to figures submitted for online review and prepublication.

Figure legends. All legends must begin with a short descriptive sentence that sums up the intent and content of the data contained in the figure. This sentence should be in boldfont. A more detailed explanation of the data contained in the figure and/or its parts should follow. The detailed description should be in Roman type (ie, not in boldfont).

Colour charges. Colour charges apply to all articles printed in *Basic & Clinical Pharmacology and Toxicology.* Therefore, please note that if there is colour artwork in your manuscript when accepted for publication, which you would like to appear in print, Blackwell Publishing require you to complete and return a colour work agreement form before your paper can be published. This form can be downloaded as PDF from the internet. (Please note that we are pleased to provide colour online only free of charge. If you would like to take advantage of this, please ensure that your image is suitable for both colour and black/white publication. For example, please do not refer to the 'red dots' or 'blue lines', as these will NOT be apparent in the print version.) The Web address for the form is: http://www.blackwellpublishing.com/pdf/SN_Sub2000_F_CoW.pdf. Once completed, please return the form to the Production Editor. Please correspond with the Editor-in-chief on acceptance of the manuscript.

References

As of 1 January 2007, references must be cited according to the Vancouver system(http://www.icmje.org).Basic & Clinical Pharmacology & Toxicology deviates from icmje in the following respects:

No full stop after the abbreviated name of the journal

As journals paginate consecutively, issue number is not used; only volume and page number.

Journal names should be abbreviated according to the system used in Index Medicus (Pubmed Service "Journals Database").
Number the references consecutively in the order they appear for the first time in the text. References that are cited in table or figure texts should be numbered in accordance with the first appearance of the table or figure in question. References in the text must be cited with the appropriate number in square bracket. In case of more than one reference in one square bracket, the numbers must be separated by a comma, e.g. [3, 4, 8]; In case of more than two consecutive reference numbers, use hyphen, e.g. [6-9];

Avoid abstracts and meeting proceedings as references; unpublished observations and personal communications must not be used as references. Accepted manuscripts that have not yet been published may appear in the reference list. Indicate journal name and year in question followed by "in press" in brackets. Articles

published online are identified by their DOI (Digital Object Identifier System) reference (http://dx.doi.org/). Electronic material is cited like other literature.

In general, references must be written as follows

Surname followed by initial(s) without comma or full stop; then comma before the next author's surname followed by his/her initial(s): Larsen JT, Brøsen K.
If there are more than six authors, et al after the sixth author's initial(s) should be added:
Smith PJ, Byron AM, Jones E, Andersson C, Tucker AD, Rowland P et al.
Put a full stop after the last author's initials (or after et al), and before the article/monography title etc.
The title should be written with initial capital while all other words are in small letters, unless it is a matter of nationalities (in a Swedish population) or names of e.g. commissions and the like. As a rule, continue with small letters after colon. Do not include subtitle.
The journal name should be written with ordinary font (no italics); do not put full stops between the individual parts of the abbreviation or between the journal name and year: Basic Clin Pharmacol Toxicol 2005.
Year, volume number and pages should be written without space.
Put a semicolon between year and volume number and colon between volume number and page number: BMJ 2004;329:1233-6.

Examples of correctly written references

Journal article
Brøsen K, Skjelbo E, Rasmussen BB, Poulsen HE, Loft S. Fluvoxamine is a potent inhibitor of cytochrome P4501A2. Biochem Pharmacol 1993;45:1211-4.
Rasmussen SG, Gether U. Purification and fluorescent labelling of the human serotonin transporter Biochemistry 2005;44:3494-505.

Book chapter
Zanger UM, Eichelbaum M. CYP2D6. In: Levy RH, Thummel KE, Trager WF, Hansten PD, Eichelbaum M (eds). Metabolic Drug Interactions. Lippincott Williams & Willis Philadelphia PA 2002;87-94.

DOI reference
Masmanian SK, Thon-That H, Schneewind O. Sortase catalysed anchoring of surface proteins to the cell wall of Stapylococcus aureus. Mol Microbiol 2001;40:1049-1057.

Doi: 10.1046/j. 1365-2958.2001.02411.x

Internet reference
http://www.imm.ki.se/CYPalleles /March 2006

We recommend the use of a tool such as EndNote or Reference Manager for reference management and formatting. EndNote reference styles can be searched

for here: http://www.endnote.com/support/enstyles.asp. Reference Manager reference styles can be searched for here: http://www.refman.com/support/rmstyles.asp.

Language
The manuscript should be written in a concise and clear British English language. Some linguistic in-house correction is performed, however, a manuscript may be returned to the authors for major linguistic revision and rewriting. The authors have the full responsibility for the English language and the style of the manuscript. The manuscript will be edited according to the style of the journal, and the proofs must be read carefully by the author.

Nomenclature
The international nomenclature should be used. Chemical formulae should as far as possible be written on one line. If proprietary names are used, the chemical constitution or, if this is not known, the outline of the preparation must appear clearly in the text. When available, INN-names should be used.
Only officially accepted abbreviations and abbreviations of long chemical names etc. should be used. Unnecessary abbreviations impede the reading of a paper; therefore the number of abbreviations should be kept at an absolute minimum. Necessary abbreviations should be used with consistency and defined at first mention. Abbreviations should not be used in the title of the paper and in the running title.

Page charges

Authors will be charged GBP 150 for each page exceeding 6 printed pages (for "Short Communications" GBP 150 for each page exceeding 2 printed pages). Authors will receive an invoice at the time of receipt of proofs.

Short communications

BASIC & CLINICAL PHARMACOLOGY & TOXICOLOGY will accept "Short Communications" for rapid publication of important experimental results and short preliminary results. The length of the manuscript should not exceed 2 printed pages including title, general text, presentation of experimental results without headings, either table or figure, and references. The general rules for manuscripts published in BASIC & CLINICAL PHARMACOLOGY & TOXICOLOGY should be followed. No abstract is necessary. Authors will be charged for pages in excess of 2 printed pages, according to the above Page charge paragraph.

MiniReviews
BASIC & CLINICAL PHARMACOLOGY & TOXICOLOGY accepts invited MiniReviews, which will be peer-reviewed and should conform to the form and style of the journal. MiniReviews should be up to 8 printed pages including a maximum of 50 references.

Letters to the Editor and Annotations & Reflections
Letters to the Editor may contain essential corrections and comments to articles published in the journal. Letters to the Editor will be published without delay after acceptance. Annotations & Reflections concerning important developments within the publication scope of the journal will be accepted by the Editor. Annotations & Reflections should not exceed 6 printed pages.

Supplements
BASIC & CLINICAL PHARMACOLOGY & TOXICOLOGY will publish supplements, consisting of e.g. monographs, abstracts or papers from a congress or a symposium, articles or a series of articles or a doctorate thesis. The publication of the supplement should be approved by the journal, and the authors or organizers will be responsible for all production and mailing costs, unless otherwise agreed. After approval, the supplements are not subject to revision by the Editorial Office. The quality of language must meet the standards maintained by the journal, and all the above-mentioned instructions should be followed. Supplements are delivered free of charge to the subscribers of the journal.
Printing should preferably be done at the printing house of the journal. Further information and cost estimates may be obtained from the Editorial Office.

Offprints
The corresponding author will be provided with a free pdf-file of the article.

Announcements
BASIC & CLINICAL PHARMACOLOGY & TOXICOLOGY will accept for publication announcements of interest to pharmacologists and toxicologists (the readers), such as notices of congresses, society meetings, symposia, awards, or other matters.