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PROPRIEDADES ANTINOCICEPTIVAS E ANTIOXIDANTES DO DISSELENETO DE BIS(FENILIMIDAZOSELENAZOLILA) EM ROEDORES

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

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2016

Dedico esta tese a minha família,
a qual me apoiou em todas as minhas decisões.

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Agradeço a Deus pelo dom da vida.

À minha família, pelo apoio em minhas decisões, pelos conselhos dados e pela força que me deram nos momentos bons e nos momentos difíceis, vocês me inspiram e espero que inspire a vocês também.

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"Pouco conhecimento faz com que as pessoas se sintam orgulhosas.
Muito conhecimento, com que se sintam humildes."
Leonardo da Vinci

RESUMO

PROPRIEDADES ANTINOCICEPTIVAS E ANTIOXIDANTES DO DISSELENETO DE BIS(FENILIMIDAZOSELENAZOLILA) EM ROEDORES

Autor: Pietro Maria Chagas
Orientadora: Dr.ª Cristina Wayne Nogueira

As drogas atualmente disponíveis para o tratamento de doenças relacionadas a dor e a inflamação apresentam inúmeros efeitos adversos, sendo assim a procura por novas moléculas é necessária. Sabe-se que compostos orgânicos de selênio possuem importantes propriedades como moléculas antioxidantes, anti-inflamatórias e antinociceptivas. O objetivo desta tese foi avaliar as propriedades antinociceptivas e antioxidantes do disseleneto de bis(fenilimidazoselenazolila) (DFIS) em roedores. Afim de atender a este objetivo, o composto foi investigado em diferentes modelos experimentais e os resultados foram divididos em dois artigos científicos e dois manuscritos. Os resultados do artigo 1 demonstraram a atividade antinociceptiva do DFIS em modelos de nocicepção térmica e química, e observou-se que nas doses testadas, o composto não induziu alterações comportamentais nem toxicidade aparente. No artigo 2, o estudo focou-se na pesquisa do potencial antioxidante do composto DFIS, onde foi reportado o efeito antioxidante *in vitro* do composto frente aos modelos de indução de peroxidação lipídica e carbonilação de proteínas em homogeneizados de cérebro, além de atividade sequestrante de radicais livres e mimética a enzimas antioxidantes. Em relação à pesquisa de sua toxicidade *in vitro*, observou-se efeito inibitório sobre a atividade das enzimas δ-aminolevulinato-desidratase e Na^+, K^+-ATPase, assim como da captação de [^{3}H]glutamato, entretanto, esses efeitos foram detectados em concentrações maiores do que as que apresentaram efeito antioxidante. *In vivo*, o DFIS também foi avaliado frente ao modelo de dano oxidativo induzido por nitroprussiato de sódio (NPS); apresentando efeito protetor contra o aumento dos níveis de peroxidação lipídica e carbonilação de proteínas e redução nos níveis de tiol não proteico, induzidos pelo NPS. No manuscrito 1, o DFIS foi avaliado frente a nocicepção inflamatória induzida pela administração intraplantar de adjuvante completo de Freund (CFA), onde a alodínia mecânica induzida por CFA foi revertida pelo composto; observou-se também que o efeito do DFIS foi bloqueado pela pré-administração de L-arginina. Em relação às análise teciduais do manuscrito 1, embora o DFIS não tenha protegido das alterações induzidas pelo CFA na pata, este protegeu do aumento dos níveis de espécies derivadas do óxido nítrico (NOx) na medula espinhal. Além disso, o DFIS também reverteu o aumento nos níveis de malondialdeído e a diminuição da captação de [^{3}H]glutamato induzidas pelo CFA na medula espinhal. No manuscrito 2, o DFIS foi avaliado frente ao modelo de artrite reumatoide induzida por colágeno tipo-II (AIC), onde foi eficaz em reverter a alodínia mecânica e hiperalgesia térmica induzidas pelo modelo. Neste protocolo, além de diminuir a atividade da enzima mieloperoxidase na pata, também diminui os níveis de NOx na medula espinhal que foram alterados pela AIC. O DFIS também reverteu o aumento dos níveis de NFκB e apresentou uma diminuição *per se* dos níveis da ciclooxigenase-II. Juntos os resultados contidos nesta tese sugerem que o DFIS é uma molécula de interesse para o desenvolvimento de futuras terapias para o tratamento de doenças relacionadas a dor e a inflamação e que a atividade antinociceptiva do DFIS está relacionada ao seu mecanismo antioxidante, principalmente a sua interferência sobre a via do óxido nítrico.

Palavras-chave: Selênio. Nocicepção. Inflamação. Artrite. Antioxidante. Óxido Nítrico.

ABSTRACT

ANTINOCICEPTIVE AND ANTIOXIDANT PROPERTIES OF BIS(PHENYLIMIDAZOSELENAZOLYL) DISELENIDE IN RODENTS

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Advisor: Cristina Wayne Nogueira, PhD

The current available drugs for treatment of disorders related to pain and inflammation present several adverse effects, then the investigation for novel molecules are required. It is known that organoselenium compounds have important properties antioxidant, anti-inflammatory and antinociceptive molecules. The objective of this thesis was to evaluate the antinociceptive and antioxidant properties of the bis(phenylimidazoselenazolyl) diselenide (BPIS) in rodent. In order to accomplish this objective, the compound was investigated in different experimental models and the results were divided in two research articles and two manuscripts. Firstly, the results of the 1st article demonstrate the antinociceptive property of BPIS in thermal and chemical nociceptive models, and it was also observed that the compound did not induce, in the tested doses, any behavioral change or apparent toxicity. In the 2nd article, the study was focused on the research of the antioxidant potential of the BPIS, and it was reported the *in vitro* antioxidant effect of the compound in front of models of lipid peroxidation and protein carbonylation induction in brain homogenates, in addition to free radical scavenger and antioxidant enzymes mimetic activity. In relation to the investigation of the *in vitro* toxicity, it was observed inhibitory effects on the activity of enzymes, such as δ-ALA-D and Na^+, K^+-ATPase, as well as [^{3}H]glutamate uptake, however, these effects were detected in higher concentrations than that the compound presented antioxidant effect. *In vivo*, BPIS was also evaluated in front of the model of oxidative damage induced by sodium nitroprusside (SNP); presenting protective effect against the increase in lipid peroxidation and protein carbonyl levels, as well as the decrease in non-protein thiols induced by SNP. In the 1st manuscript, BPIS was evaluated in the inflammatory nociception induced by intraplantar injection of complete Freund's adjuvant (CFA), where the mechanical allodynia induced by CFA was reversed by the compound; it was also seen that the BPIS effect was blocked by L-arginine pre-administration. In relation to the tissue analysis of the 1st manuscript, though BPIS did not protected against the changes induced by CFA in the paw tissue, it protected against the increase in the nitric oxide related species (NOx) in the spinal cord. Besides that, BPIS also reversed the augment in malondialdehyde levels and reduction in [^{3}H]glutamate uptake induced by CFA in the spinal cord. In the 2nd manuscript, BPIS was evaluated in front of the type-II collagen-induced rheumatoid arthritis model (CIA), where it was effective in reversing the mechanical allodynia and thermal hyperalgesia induced by the model. In this protocol, BPIS decreased the paw myeloperoxidase activity, as well as the NOx levels in the spinal cord, that were altered by CIA. BPIS also blocked the increase in NFκB levels and induced a *per se* decrease in cyclooxygenase-II levels. Together, the results in this thesis suggest that BPIS is a molecule of interest to the development of novel therapies for the treatment of disorders related pain and inflammation, and that BPIS antinociceptive property is related to its antioxidant mechanism, mainly its interference on the nitric oxide pathway.

Keywords: Selenium. Nociception. Inflammation. Arthritis. Antioxidant. Nitric Oxide.

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LISTA DE ABREVIATURAS

$(ClPhSe)_2$ – Disseleneto de *p*-cloro-difenila

$(F_3CPhSe)_2$ – Disseleneto de *m*-trifluorometil-difenila

$(MeOPhSe)_2$ – Disseleneto de *p*-metoxi-difenila

$(PhSe)_2$ – Disseleneto de difenila

3-NT – 3-nitrotirosina

5-HT – Serotonina

AIC – Artrite reumatoide induzida por colágeno tipo-II de frango

AINE – Anti-inflamatório não esteroidal

ALT – Alanina aminotransferase

ASICs – Canais iônicos sensíveis a ácido

AST – Aspartato aminotransferase

BHE – Barreira hematoencefálica

CAT – Catalase

CFA – Adjuvante completo de Freund

CGRP – Peptídeo relacionado ao gene da calcitonina

CID – Classificação Internacional de Doenças e Problemas relacionados à Saúde

CI_{50} – Concentração inibitória 50%

COX – Ciclooxigenase

DARMD – Drogas antirreumáticas modificadoras de doença

DFIS – Disseleneto de bis(fenilimidazoselenazolila)

DHAR – Deidroascorbato redutase

DNA – Ácido desoxirribonucleico

eNOS – Óxido nítrico sintase endotelial

ERN – Espécie reativa de nitrogênio

ERO – Espécie reativa de oxigênio

EUA – Estados Unidos da América

GLT-1 – Transportador de Glutamato-1

GPx – Glutationa peroxidase

GR – Glutationa redutase

GSH – glutationa

GST – Glutationa-S-transferase

HIF – Fator induzido por hipóxia

IASP – Associação Internacional para o Estudo da Dor

i.c.v. – Intracerebroventricular

IL – Interleucina

iNOS – Óxido nítrico sintase induzível

LOOH – Hidroperóxido lipídico

LOX – Lipoxigenase

MAPK – Proteína quinase ativada por mitógeno

MDA – Malondialdeído

MPO – Mieloperoxidase

NA – Noradrenalina

NAPHox – Nicotinamida adenina dinucleotídeo fosfato oxidase

NFκB – Fator nuclear kapa-B

nNOS – Óxido nítrico sintase neuronal

NOx – Espécies relacionadas ao óxido nítrico

NPS – Nitroprussiato de sódio

NPSH – Tiol não-proteico

PI3K – Fosfatidilinositol-3-quinase

PLA2 – Fosfolipase A2

SNC – Sistema Nervoso Central

SOD – Superóxido dismutase

TGF-β – Fator de transformação do crescimento-beta

TNF-α – Fator de necrose tumoral-alfa

TRPV1 - Receptores de potencial transitório do tipo vaniloide

SUMÁRIO

1 INTRODUÇÃO

1.1 DOR

A dor, embora seja uma experiência complexa e difícil de ser descrita em um simples conceito, é definida pela Associação Internacional para o Estudo da Dor (IASP) como uma “experiência sensorial ou emocional desagradável associada com real ou potencial dano tecidual ou descrita em termos de tal dano” (LOESER e TREEDE, 2008). Portanto, entende-se que a percepção da dor não envolve apenas a tradução de um estímulo nocivo (processo chamado de nocicepção), mas também processos emocionais e cognitivos no cérebro, podendo não ser sempre idêntica e sofrer variáveis que contribuem na experiência pessoal (JULIUS e BASBAUM, 2001). Enquanto a nocicepção pode ser definida como a detecção de estímulos nocivos e a consequente transmissão da informação codificada ao cérebro, a dor é essencialmente um processo perceptual que origina-se em resposta a tal evento (LOESER, 2000).

A transmissão do estímulo nociceptivo envolve inicialmente a ativação de neurônios sensoriais ou aferentes primários, através de uma classe de receptores, também conhecida como receptores nociceptivos, sendo esses neurônios diferenciados pelo diâmetro das fibras condutoras de seus axônios, como demonstrado na Figura 1 (JULIUS e BASBAUM, 2001).

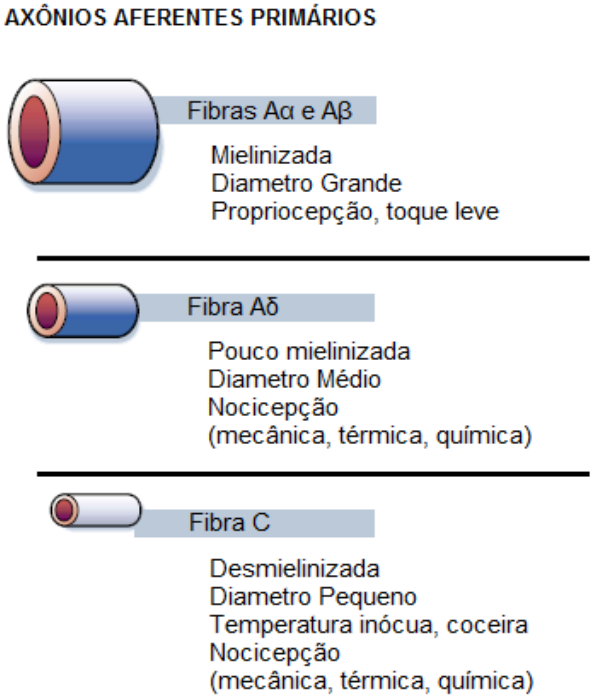

Figura 1. Características das fibras de aferentes primárias. Adaptado de JULIUS e BASBAUM, 2001.

Os estudos em neurofisiologia também salientam diferenças na transmissão de estímulos nocivos e não-nocivos pelos neurônios sensoriais primários: sob condições normais, fibras de limiar baixo e alta velocidade de condução (fibras Aα e Aβ) possuem receptores especializados encapsulados e são estimuladas por estímulos não-nocivos; por outro lado, fibras de limiar alto e menor velocidade de condução (fibras Aδ e C) possuem terminações livres e são estimuladas por estímulos nocivos através dos receptores nociceptivos (SCHAIBLE e GRUBB, 1993). Dentre os receptores nociceptivos, podemos citar alguns receptores ionotrópicos relacionados a transdução sensorial, como os receptores vaniloides, receptores sensíveis a prótons e produtos do metabolismo purinérgico (DUBIN e PATAPOUTIAN, 2010).

Como mencionado anteriormente a sensação nociceptiva aguda é resultante da ativação de neurônios sensoriais primários, constituídos de fibras de limiar alto (fibras Aδ e C) e dão início a transmissão desta informação a medula espinhal que, por sua vez é retransmitida a neurônios que se projetam contralateralmente a níveis supraespinhais, como o tálamo e o córtex somatossensorial, sendo este processo modulado por uma ampla gama de mediadores (HOLMES, 2016; MILLAN, 1999), como representado na Figura 2.

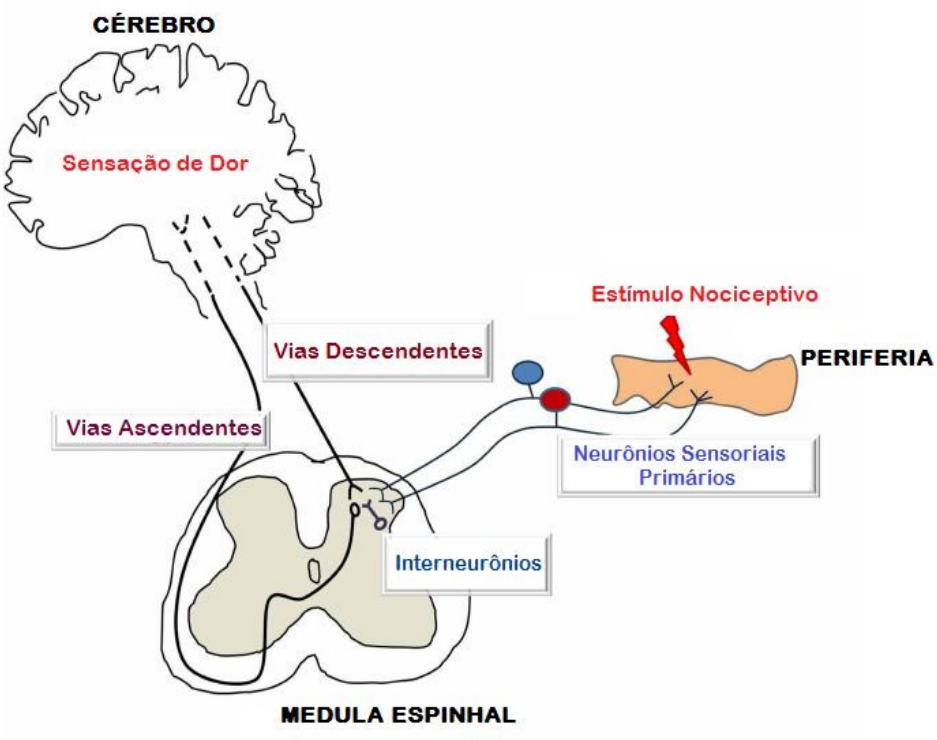

Figura 2. Representação esquemática da principal via neuronal ascendente e o sistema modulatório da via descendente responsáveis pela dor. Adaptado de TAVARES e MARTINS, 2013.

Do ponto de vista molecular, os neurônios sensoriais primários podem ser ativados por diversos fatores (ex. lesão tecidual, queimaduras ou processos inflamatórios), liberando a nível espinhal inúmeros neurotransmissores, como aminoácidos excitatórios glutamato e aspartato, peptídeo relacionado ao gene da calcitonina (CGRP), substância P, galanina e neuropeptídio Y. A transmissão da informação nociceptiva a nível espinhal é modulada por interneurônios os quais podem liberar peptídeos opioides e ácido γ-aminobutírico, assim como também é modulada por neurônios supraespinhais (vias descendentes) através da liberação de serotonina (5-HT) e noradrenalina (NA). Esses mecanismos modulatórios podem inibir ou facilitar a transmissão nociceptiva através da medula espinhal (TAVARES e MARTINS, 2013). Uma vez que sinais são transmitidos através dos receptores nociceptivos ao sistema nervoso central (SNC), estas informações são processadas, gerando inúmeras respostas fisiológicas e comportamentais. Dentre as respostas geradas podemos citar a “experiência emocional desagradável”, assim como reflexos de retirada, aumento da pressão sanguínea e frequência cardíaca, entre outros parâmetros (MILLAN, 1999).

A dor, primariamente, constitui um mecanismo de alerta, o qual comunica ao indivíduo que algo está errado sempre que algum tecido for lesionado, fazendo com que este reaja para remover o estímulo promotor de dor (JULIUS e BASBAUM, 2001). Em termos de duração, a dor pode ser aguda ou crônica. A dor aguda é uma resposta normal causada por uma lesão do tecido com consequente ativação de receptores nociceptivos no local da lesão, sendo que a dor geralmente desaparece até mesmo antes do restabelecimento do tecido lesado. Por fim, a dor crônica é causada por uma lesão tecidual ou doença que geralmente ultrapassa o tempo de recuperação do organismo, ou seja, este tipo de dor não desaparece mesmo quando o trauma inicial foi resolvido, podendo durar meses ou anos, sendo um importante fator de incapacidade e sofrimento (LOTSCH e GEISSLINGER, 2001; MELZACK, 1999). A dor crônica, por si só, pode ser considerada como uma doença incapacitante, presente em muitas condições médicas e o controle da dor constitui uma importante prioridade terapêutica (PHILLIPS e CLAUW, 2011). A dor crônica também pode ser dividida em dor inflamatória e dor neuropática de acordo com a sua etiologia. A dor inflamatória é relacionada a estímulos periféricos, nos quais um dano ou um processo inflamatório leva a ativação de uma cascata imune relacionada a liberação de mediadores inflamatórios locais, derivados do sangue ou

também de células imunes ou danificadas, os quais levam a ativação de receptores presentes em fibras C; embora a dor inflamatória possa ser resolvida com a resolução do dano tecidual, isto dificilmente é conseguido, uma vez que na maioria das vezes a dor está relacionado a processos inflamatórios crônicos e progressivos. Enquanto isso, a dor neuropática é relacionada a dano aos nervos periféricos, levando a sensação de dor espontânea contínua devido a ativação das vias aferentes relacionados ao axônio danificado, a qual permanece ainda que o dano inicial seja resolvido. Além das diferenças quanto a etiologia, os diferentes tipos de dor crônica apresentam também tratamentos diferenciados; sabe-se que a dor inflamatória responde geralmente a agentes anti-inflamatórios e drogas opioides, enquanto a dor neuropática responde a agentes antidepressivos e anticonvulsivantes (XU e YAKSH, 2011).

Uma vez que a simples dor aguda já caracteriza uma experiência desagradável, situações de dor crônica são de grande preocupação. Estudos demonstram que em países desenvolvidos a dor crônica afeta em torno de 20% da população adulta. Em torno de 30-40% dos acometidos pela dor crônica estão relacionados a doenças das articulações e musculoesqueléticas; seguidos por dor nas costas e pescoço que contabilizam por outros 30% e enxaqueca e cefaleias contabilizam em torno de 10% dos casos de dor persistente. Além disso, embora em menor número, em torno de 1-2% dos adultos também são afetados por dor resultante de câncer (BREIVIK e BOND, 2004; STEGLITZ et al., 2012). Sabe-se que nos Estados Unidos da América (EUA), gasta-se em torno de US$ 600 bilhões com custos diretos com medicamentos, comorbidades e perda de produtividade dos pacientes; sendo destes, em torno de 30% envolvidos exclusivamente com patologias relacionadas a artrite (HOLMES, 2016). A Figura 3 apresenta os principais sinais e sintomas relacionados a dor crônica como uma doença *per se* (Classificação Internacional de Doenças e Problemas relacionados à Saúde – CID-10 R52), assim como suas comorbidades.

Não é apenas a duração que distingue a dor aguda da dor crônica, mas a capacidade do organismo de reparar o sítio da lesão e restaurar os disparos aferentes e o processamento central normal (LOESER, 2000). Tendo em vista que estes eventos são dependentes da intensidade e da duração do estímulo, quanto mais persistente for o processo doloroso, mais difícil se torna o tratamento do quadro patológico (BESSON e CHAOUCH, 1987; WANG e WANG, 2003; WOOLF e

SALTER, 2000; ZIMMERMANN, 2001). Situações de dor, principalmente de cunho crônico, podem estar combinadas a mudanças neuroplásticas no SNC que podem levar à sensibilidade aumentada a dor: condições conhecidas como hiperalgesia, ou aumento da dor em resposta a estímulos dolorosos; e alodínia, ou dor em resposta a estímulos não-nociceptivos (LOESER e TREEDE, 2008).

Sinais e Sintomas de Dor Crônica, uma vez que esta tenha evoluído para uma doença *per se*
1. Imobilidade e consequente desgaste de músculos, articulações, etc.
2. Depressão do sistema imune e aumento da susceptibilidade a doenças
3. Sono perturbado
4. Perda de apetite e desnutrição
5. Dependência em medicamentos
6. Dependência exagerada de familiares e outros cuidadores
7. Uso abusivo e inapropriado de sistemas de saúde
8. Piora da performance no trabalho ou incapacidade
9. Isolamento da sociedade e familiares
10. Ansiedade e medo
11. Amargura, frustração, depressão e tentativas de suicídio

Figura 3. Principais sinais e sintomas relacionados a dor crônica como uma doença *per se* (CID-10 R52), assim como suas comorbidades. Adaptado de BREIVIK e BOND, 2004.

Além disso também é importante lembrar que mesmo em pacientes que apresentam dor com componentes mais periféricos, e não primariamente neuropáticos, como osteoartrite e artrite reumatoide, podem frequentemente ter elementos relacionados a hipersensibilidade a dor a nível de SNC (PHILLIPS e CLAUW, 2011). Cabe salientar também que tanto a dor aguda quanto a dor crônica estão frequentemente associadas a processos inflamatórios, como resultado da lesão tecidual, reatividade imunológica anormal ou lesão nervosa (STEIN et al., 2003).

1.2 INFLAMAÇÃO

A inflamação, assim como a dor, constitui um mecanismo fisiológico de defesa sendo estes dois muitas vezes processos relacionados, os quais partilham de efetores e mediadores comuns. Reforçando esta afirmativa, cabe lembrar que um dos sinais clássicos do processo inflamatório em si, é justamente a dor, sendo os outros calor, rubor, inchaço e consequente perda da função do órgão/local afetado (LAWRENCE et al., 2002). Muito antes de uma patologia, a inflamação é uma resposta do sistema imunológico, a qual pode ser causada por diversos agentes

químicos, físicos e infecciosos envolvendo recrutamento e a ativação de leucócitos para o sítio tecidual afetado; podendo o processo inflamatório também estar relacionado a um desequilíbrio no controle imunológico celular (BRUNE et al., 2013).

A nível primário, a inflamação desencadeada pelos agentes anteriormente mencionados envolve a chegada de diversos componentes sanguíneos, como plasma e leucócitos ao local afetado. A resposta inicial é local e mediada por células tecido-residentes como macrófagos e mastócitos, levando a produção de diversos mediadores inflamatórios, incluindo citocinas, aminas vasoativas, eicosanoides, moléculas gasosas, entre outros (MEDZHITOV, 2008). A partir da produção destes mediadores, o efeito principal e mais imediato é a formação de exsudato inflamatório local, onde proteínas plasmáticas e leucócitos, normalmente restritos ao sangue ganham acesso ao tecido afetado, possivelmente devido a alterações na permeabilidade do endotélio vascular e a mecanismos quimiotáticos (POBER e SESSA, 2007).

Quando os leucócitos alcançam o local afetado, estes tornam-se ativados por mecanismos relacionados à interação direta com os patógenos ou devido a ação de citocinas secretadas pelas células residentes no tecido. Uma vez ativados, os leucócitos, principalmente os neutrófilos, tentam eliminar o insulto inicial, como os agentes infecciosos, liberando o conteúdo tóxico presente em seus grânulos (FAURSCHOU e BORREGAARD, 2003). Estas substâncias, embora sejam extremamente potentes e necessárias para a eliminação de patógenos, não discriminam entre micro-organismos e os alvos do próprio hospedeiro, assim, danos colaterais aos tecidos do hospedeiro podem ocorrer. Uma resposta inflamatória aguda satisfatória e bem sucedida resulta na eliminação do insulto inicial seguida de uma fase de resolução e reparo (DE OLIVEIRA et al., 2016).

Reforçando o anteriormente mencionado, neutrófilos são uma das classes de leucócitos mais importantes para o desenvolvimento do processo inflamatório local, além de serem o tipo mais abundante de leucócitos no sangue e uma das primeiras linhas de defesa do organismo contra infecções e dano tecidual. Os neutrófilos realizam sua ação como células efetoras destruindo agentes infecciosos através de processos como fagocitose, degranulação, assim como ajudando a regular a resposta adaptativa imune, incluindo a ativação de linfócitos B e T (CROCKETT-TORABI e WARD, 1996). Em uma resposta bem sucedida a um dano agudo, é fundamental que uma lesão tecidual muito extensa, decorrente da resposta

imune, seja prevenida através da resolução do processo inflamatório e remoção dos neutrófilos do local onde ocorreu o dano. Embora a participação destas células sejam de extrema importância para a resposta imune, infiltração e ativação excessiva de neutrófilos no local de ação também pode levar a dano tecidual e consequente inflamação crônica (DE OLIVEIRA et al., 2016).

Diversas são as substâncias e enzimas presentes nos grânulos de células leucocitárias, as quais estão relacionadas a produção de toxinas ou moléculas sinalizadoras relacionadas a destruição de possíveis patógenos. Dentre os produtos que ativam diretamente o processo inflamatórios estão: citocinas, quimiocinas, proteases, prostaglandinas, leucotrienos, espécies reativas de oxigênio (EROs) e nitrogênio (ERNs) (WRIGHT et al., 2010) (Figura 4). Para a produção de algumas destas substâncias são necessárias certas enzimas como: a fosfolipase A2 (PLA2), ciclooxigenases (COX) e as lipooxigenases (LOX), responsáveis pela produção de prostaglandinas e leucotrienos, respectivamente; e a nicotinamida adenina dinucleotídeo fosfato oxidase (NAPHox), a mieloperoxidase (MPO) e a óxido nítrico sintase induzível (iNOS), responsáveis pela produção de superóxido, ácido hipocloroso e óxido nítrico, respectivamente (SEGAL, 2005; WRIGHT et al., 2010). Frequentemente, a expressão ou mesmo a atividade dessas enzimas, assim como os níveis dos produtos, estão aumentados tanto em situações de inflamação aguda ou crônica, sendo utilizados como marcadores de atividade inflamatória tecidual (FEITOZA et al., 2009; TAHERGORABI e KHAZAEI, 2013; ZHOU et al., 2004).

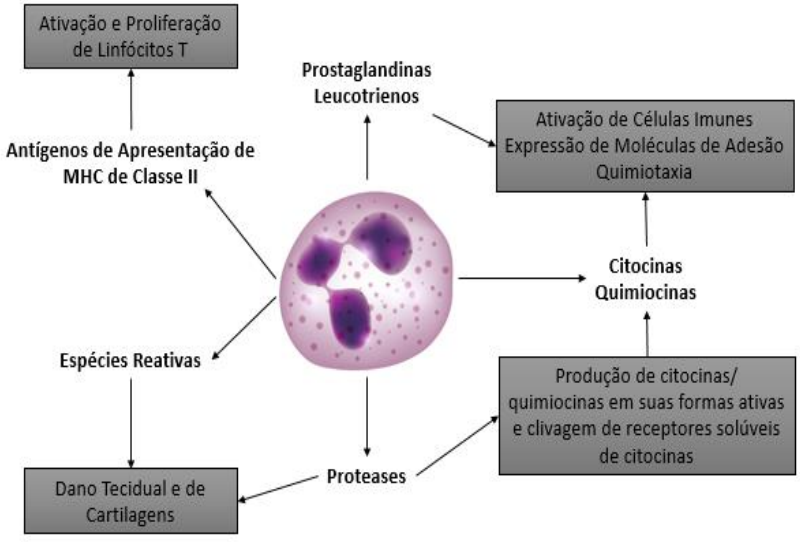

Figura 4. Funções e produtos de leucócitos que ativam diretamente o processo inflamatório. Adaptado de WRIGHT et al., 2010.

A resposta inflamatória é orquestrada por diversos mediadores, sendo um dos principais deles as citocinas, produzidas por diversos tipos celulares. Apesar de a natureza do estímulo nocivo geralmente ditar os tipos de mediadores produzidos, alguns estão presentes de forma quase obrigatória nos diversos processos, dentre eles podemos citar o fator de necrose tumoral-alfa (TNF-α) e as interleucinas (IL)-1 e IL-6 (MEDZHITOV, 2008). Estas citocinas, secretadas inicialmente pelas células tecido-residentes, tem função basicamente pró-inflamatória, são responsáveis por funções como ativação endotelial e leucocitária e indução da resposta de fase aguda (FOSTER, 2001; KIELIAN e HICKEY, 2000). O TNF-α e as interleucinas IL-1 e IL-6, agindo sobre os seus receptores celulares específicos, ativam diversas vias inflamatórias, sendo uma das principais a via do fator nuclear-kapa-B (NF-κB), um complexo proteico que desempenha funções como fator de transcrição de genes relacionados a síntese de enzimas e outros mediadores inflamatórios (LAWRENCE, 2009). Após a retirada do insulto inicial, geralmente ocorrem os fenômenos de recirculação e apoptose de células sanguíneas, assim como a diminuição da produção dos mediadores inflamatórios (ex. eicosanoides, quimiocinas e citocinas pró-inflamatórias) (LAWRENCE e GILROY, 2006). Assim como existem citocinas com função pró-inflamatória, não se pode esquecer que também existem algumas com função anti-inflamatória, dentre as principais podemos citar a IL-10 e o fator de transformação do crescimento-β (TGF-β), as quais desempenham papéis importantes na resolução da inflamação e iniciação do reparo tecidual (COUPER et al., 2008; LAWRENCE et al., 2002; MEDZHITOV, 2008).

Como anteriormente citado, a inflamação geralmente é caracterizada como uma resposta de cura a uma injúria local, entretanto esta pode evoluir para estados crônicos caso o insulto inicial persista, seja ele um antígeno endógeno ou exógeno, elevando o risco de uma progressão para um resposta sistêmica (MEDZHITOV, 2008). Um dos principais exemplos de doenças inflamatórias crônicas são as doenças autoimunes, das quais podemos citar a esclerose múltipla, a diabetes tipo I, a artrite reumatoide e a doença celíaca, mas também não podem ser excluídas doenças onde o papel autoimune não está necessariamente presente, como a asma, diabetes tipo II, aterosclerose e doença inflamatória do intestino (HEAP e VAN HEEL, 2009). Ainda não é totalmente compreendido os mecanismos pelos quais tanto o organismo poderia atacar as próprias células ou persistir demasiadamente em um processo inflamatório exacerbado que acabaria por causar

mais estragos que soluções. O organismo provavelmente falhe em ativar certos genes, redes e vias de sinalização necessárias para cessar a inflamação crônica de uma forma coordenada (HEAP e VAN HEEL, 2009; LAWRENCE e GILROY, 2006).

Lembrando o anteriormente citado, a dor é uma das características clássicas do processo inflamatório, e o contrário também se observa em situações de dor, como uma doença *per se*, onde a inflamação tem um papel importante na propagação e sensibilização de receptores a estímulos nociceptivos (KIDD e URBAN, 2001). Mediadores inflamatórios, como prostaglandinas e citocinas, de forma aguda são conhecidos por ativarem ou aumentarem a sensibilidade de receptores nociceptivos a determinados agonistas (HORI et al., 2000; HORI et al., 1998). Além disso, estudos demonstram que situações inflamatórias, mesmo periféricas, podem desencadear liberação de mediadores inflamatórios de uma forma sistêmica, podendo culminar até mesmo em processos neuroinflamatórios, com ativação de células imunes cerebrais, ou microglias, presentes tanto no cérebro quanto na medula espinhal (RAGHAVENDRA et al., 2004; SWEITZER et al., 1999). A ativação destas células, junto com uma maior sensibilidade de outras células neuronais, como neurônios e astrócitos, podem contribuir extensamente na patofisiologia da dor crônica (GOSSELIN et al., 2010; MILLIGAN e WATKINS, 2009). Mesmo em situações de dor crônica em que o processo inflamatório não é a causa inicial, como em dores neuropáticas, comenta-se que ocorre também a ativação neuroimune, assim como um aumento na produção de citocinas e quimiocinas a nível de SNC (KIDD e URBAN, 2001; PHILLIPS e CLAUW, 2011).

Dentre as doenças em que dor e inflamação estão amplamente correlacionadas está a artrite reumatoide: um doença de cunho autoimune que afeta inúmeras pessoas, e que apresenta associação com incapacidade progressiva, complicações sistêmicas, morte prematura e custos socioeconômicos. Sua causa ainda não é totalmente conhecida e o seu prognóstico é geralmente desfavorável, com poucas terapias efetivas e com efeito em longo prazo (MCINNES e SCHETT, 2011). Os indivíduos afetados pela artrite reumatoide apresentam processo inflamatório exacerbado nas articulações, também chamado de sinovite, caracterizado por dor e inchaço, sendo este processo crônico e incurável, o que pode levar mesmo a perda da função das mesmas (CHOY, 2012; PICERNO et al., 2015). Os principais anticorpos presentes no desenvolvimento desta patologia são os anticorpos anti-peptídio citrulinado cíclico, anti-queratina e o fator reumatoide

(principalmente do tipo Imunoglobulina M anti-Imunoglobina G), os quais originam o processo inflamatório sinovial, ainda sem causas conhecidas, com liberação de citocinas e enzimas que levam a destruição do tecido sinovial (AMRI et al., 2011; BAS, S., 2002). Embora o processo inicial apresente características especialmente retidas as articulações, manifestações sistêmicas também podem ser observadas, como vasculite, nódulos viscerais, síndrome de Sjögren e até mesmo processos inflamatórios a nível de SNC (BATHON et al., 1989; COJOCARU et al., 2010). Como uma condição associada a dor crônica, a artrite reumatoide também deve ser considerada como um “estado de dor mista”, apresentando componentes tanto centrais quanto periféricos desempenhando determinados papeis na sinalização da dor. E devido a isto, os componentes centrais devem ser levados em conta tanto na abordagem farmacológica quanto não-farmacológica deste tipo de dor, uma vez que algumas drogas são efetivas para o tratamento da dor periférica mas não central (PHILLIPS e CLAUW, 2011).

1.3 TRATAMENTOS FARMACOLÓGICOS DISPONÍVEIS PARA O TRATAMENTO DE DOENÇAS RELACIONADAS A DOR E INFLAMAÇÃO

Como anteriormente comentado, a dor é classificada quanto ao seu tempo de duração em aguda e crônica, e assim também é diferenciado seu tratamento. Um ponto importante também a ser levado em conta é que dores crônicas geralmente possuem componentes centrais ou inflamatórios associados (PHILLIPS e CLAUW, 2011).

Para o tratamento da dor aguda, os principais aspectos a serem avaliados são a causa e também a intensidade da dor relatada pelo paciente. Para o tratamento de dores leves a moderadas são usados anti-inflamatórios não esteroidais (AINEs) (BLONDELL et al., 2013). O tipo de AINE utilizado geralmente leva em conta o tipo de dor e os riscos individuais de cada paciente, uma vez que esta classe de drogas frequentemente é associada a alguns efeitos adversos, sendo os principais os gastrointestinais, devido à falta de seletividade para a isoforma induzível, ou COX-2, da ciclooxigenase. Ainda assim, mesmo fármacos inibidores seletivos para a COX-2, como os membros da classe dos coxibes, precisam ser utilizados com cautela, uma vez que estão relacionados a riscos cardiovasculares, além de serem de maior custo para o paciente (BACCHI et al., 2012). Em caso destas drogas não serem suficientes para o alívio da dor, ainda pode ser usada uma

combinação de opioides e AINEs. Dores agudas mais severas podem ser tratadas com opioides potentes, sendo esses fármacos geralmente eficazes em muitos tipos de dores (BLONDELL et al., 2013). Entretanto, para os fármacos opioides, diversos fatores devem ser levados em conta, os principais deles são o risco de desenvolvimento de tolerância e dependência, os quais devem ser considerados mesmo em tratamentos de curto prazo (BENYAMIN et al., 2008).

Para o alívio de processos inflamatórios agudos, além dos AINEs anteriormente mencionados, utilizam-se também fármacos corticosteroides. Sabe-se que os fármacos corticoides, embora apresentem grande eficácia dependendo do tratamento, também apresentam efeitos colaterais, os quais podem ser muito graves dependendo da potência do medicamento. Os corticosteroides são associados a inúmeros efeitos adversos, dos quais incluem risco aumentado de infecções, intolerância à glicose, retenção de sódio, edema e hipertensão, dificuldade de cicatrização, entre outros (SHAIKH et al., 2012).

Reforçando o supradito, doenças relacionadas a dor crônica precisam ter levados em conta os componentes centrais ou inflamatórios associados. Neste tópico serão abordados os fármacos relacionados a artrite reumatoide, por esta apresentar componentes de dor crônica de aspecto inflamatório. Para o tratamento desta doença, podem ser utilizadas drogas com características imunossupressoras, os quais são escolhidos com base no estágio da artrite. Dentre os fármacos imunossupressores mais utilizados são os corticosteroides e a classe conhecida como drogas antirreumáticas modificadoras de doença (DARMD), sendo alguns exemplos o metotrexato, os sais de ouro, a hidroxicloroquina e a sulfassalazina, além dos os inibidores biológicos do TNF-α (SINGH et al., 2016). Como complicações do tratamento dessas drogas, os agentes imunossupressores suprimem o sistema imune agindo por diversas vias, o que acaba levando a um risco elevado de infecções por diferentes microorganismos, sendo infecções bacterianas e virais as de maior preocupação. Devido ao papel do sistema imune no controle de células malignas, os pacientes podem apresentar risco teórico maior de desenvolvimento de tumores (KAHLENBERG e FOX, 2011).

Por isto, observa-se que os medicamentos disponíveis na clínica para prescrição analgésica e anti-inflamatória, embora eficazes, ainda apresentam diversas preocupações quanto aos seus efeitos adversos e segurança (JAGE, 2005;

STEINMEYER, 2000). Por essa razão, há um crescente interesse na busca de novas drogas analgésicas promissoras e com menos efeitos adversos.

1.4 ESTRESSE OXIDATIVO

Espécies reativas são moléculas eletrofílicas que apresentam certo grau de reatividade com moléculas orgânicas, sendo chamados também de radicais livres quando possuem um ou mais elétrons desemparelhados em um orbital atômico ou molecular (CADENAS e DAVIES, 2000). Fisiologicamente, as principais espécies reativas produzidas são divididas em EROs e ERNs, as quais podem ser detoxificadas por mecanismos fisiológicos como apresentados na Figura 6 (SORG, 2004).

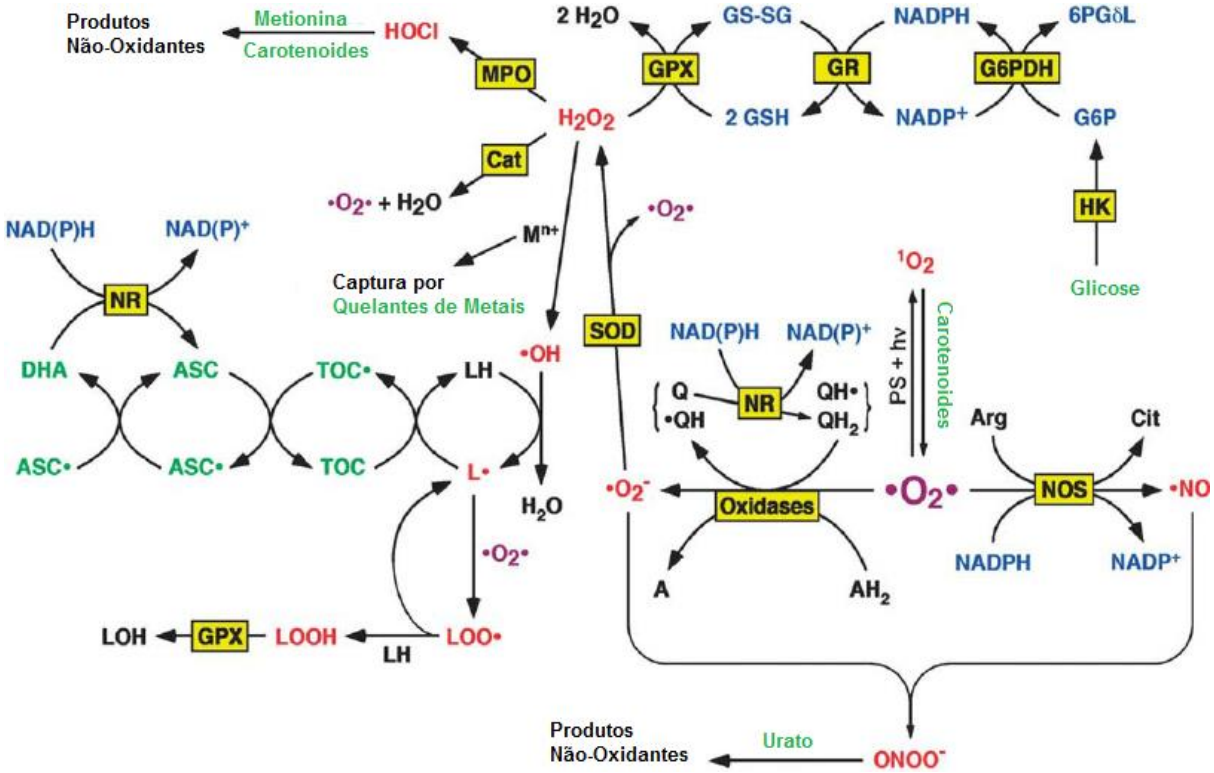

Figura 5. Representação das principais vias de síntese e eliminação de EROs e ERNs. Abreviaturas presentes na figura: 6-fosfogliconato (6PGδL), Arginina (Arg), Áscorbato (ASC), Catalase (Cat), Citrulina (Cit), Deidroascorbato (DHA), Glicose-6-fosfato (G6P), Glicose-6-Fosfato Desidrogenase (G6PDH), Glutationa Oxidada (GS-SG), Glutationa Peroxidase (GPx), Glutationa Redutase (GR), Glutationa Reduzida (GSH), Hexoquinase (HK), Lipídios e derivados (L), Metal (M), Mieloperoxidase (MPO), Nucleotídeo Redutase (NR), Óxido Nítrico Sintase (NOS), Tocoferol (TOC), Substratos diversos (A), Superóxido Dismutase (SOD) e Ubiquinona e derivados (Q). Adaptado de SORG, 2004.

Dentre as EROs podemos citar os radicais superóxido ($O_2^{\cdot -}$), hidroxila ($OH^{\cdot}$), assim como as espécies não-radicalares como peróxido de hidrogênio (H_2O_2), ácido hipocloroso (HOCl) e peróxidos lipídicos (LOOH) (VALKO et al., 2007). Estas

moléculas são produzidas fisiologicamente em pequenas quantidades, por exemplo: o radical superóxido é produzido através da redução do oxigênio molecular (O_2) mediada por enzimas como a NADPH oxidase e a xantina oxidase, assim como pela reação com a semi-ubiquinona na cadeia transportadora de elétrons mitocondrial; este superóxido pode sofrer ação da superóxido dismutase (SOD) e produzir o peróxido de hidrogênio; o peróxido de hidrogênio por sua vez pode sofrer ação de enzimas como catalase (CAT) e glutationa peroxidase (GPx) e ser detoxificado até água (H_2O) e oxigênio molecular, ou sofrer ação da enzima MPO em leucócitos e produzir ácido hipocloroso ou ainda reagir com íons metálicos, principalmente Fe^{2+}, e produzir radical hidroxila (CADENAS e DAVIES, 2000; SORG, 2004). Estas EROs podem reagir com macromoléculas celulares, principalmente lipídios insaturados, e formarem peróxidos lipídicos, os quais também podem ser detoxificados pela GPx ou chegarem até a formação do produto final malondialdeído (MDA) (AYALA et al., 2014).

Dentre as ERNs podemos citar o óxido nítrico ($NO^{\cdot}$), o peroxinitrito ($ONOO^-$) e seus derivados. O oxido nítrico é formado fisiologicamente pela enzima óxido nítrico sintase, a qual se apresenta como três isoformas: óxido nítrico sintase endotelial (eNOS), óxido nítrico sintase neuronal (nNOS) e óxido nítrico sintase induzível (iNOS); o óxido nítrico em concentrações fisiológicas possuem importantes funções como: vasodilatador, neurotransmissor, assim como apresenta um importante papel no sistema imune na destruição de patógenos (COLEMAN, 2001; PRAST e PHILIPPU, 2001). O óxido nítrico pode apresentar seus efeitos agindo principalmente sobre duas vias: como ativador da guanilato ciclase solúvel e aumentando os níveis de guanosina monofosfato cíclico (GMPc); e como agente causador de mudanças pós-traducionais através de S-nitrosilação ou nitração (RAJU et al., 2015). Entretanto, em determinadas situações inflamatórias, o óxido nítrico é produzido em quantidades maiores que as necessárias, podendo atuar como uma espécie reativa. Além disso, o óxido nítrico juntamente com o ânion superóxido, pode levar à formação da molécula de peroxinitrito, o qual também é responsável por muitas das funções tóxicas e pró-inflamatórias do óxido nítrico (CALABRESE et al., 2007). Estudar os níveis de óxido nítrico em fluídos biológicos é particularmente difícil devido a sua curta meia-vida: ao invés disso, muitas vezes são dosados seus derivados: nitrito (NO_2^-) e nitrato (NO_3^-), também conhecidos como NOx, formados

através da oxidação do óxido nítrico; e a 3-nitrotirosina, formada através da nitração de proteínas (KELM, 1999).

Altos níveis dessas espécies reativas podem causar danos a macromoléculas, como lipídios, proteínas e ácidos nucleicos, levando a alterações na função das mesmas e levando a efeitos deletérios (CADENAS e DAVIES, 2000). Por este motivo, os organismos animais apresentam maneiras para controlar os níveis de espécies reativas, as quais podem ser divididas em enzimáticas e não-enzimáticas (SCHULZ et al., 2000). Dentre as formas não-enzimáticas podemos citar as vitaminas ácido ascórbico e tocoferol, os tióis como a glutationa reduzida (GSH) e os carotenoides, e até mesmo o óxido nítrico e o urato em pequenas concentrações, entre outros: e o principal mecanismo de ação é agir como "*scavengers*" ou sequestradores de radicais livres (RIZZO et al., 2010). E como exemplo de enzimas antioxidantes podemos mencionar a CAT e a SOD, e as enzimas do ciclo da glutationa: GPx, glutationa redutase (GR), glutationa-S-transferase (GST), responsáveis tanto por detoxificar espécies reativas quanto manter os níveis adequados de antioxidantes (MOURA et al., 2015; SORG, 2004).

Em situações onde há um desequilíbrio entre a produção de EROs/ERNs e os antioxidantes naturais ocorre o chamado estresse oxidativo, o qual pode levar a danos celulares (KOHEN e NYSKA, 2002). Além dos danos diretos as macromoléculas, as espécies reativas também podem ativar cascatas de sinalização que também estão envolvidas em processos inflamatórios, como: via do NFkB, proteína quinase ativada por mitógeno (MAPK), fator induzido por hipóxia (HIF) e fosfatidilinositol-3-quinase (PI3K) (Figura 7) (SCHIEBER e CHANDEL, 2014). Estudos tem demonstrado o envolvimento do estresse oxidativo no envelhecimento e na patofisiologia de doenças como processos neurodegenerativos, câncer, aterosclerose, hepatopatias, entre outras (DALLEAU et al., 2013; HOLBROOK e IKEYAMA, 2002; HUBER et al., 1991).

Processos inflamatórios, principalmente crônicos, quando exacerbados também estão relacionados ao fenômeno do estresse oxidativo. O aumento da proliferação e ativação de células inflamatórias leva a um aumento da atividade das anteriormente mencionadas iNOS, NADPHox e MPO, as quais produzem respectivamente óxido nítrico, superóxido e ácido hipocloroso (MITTAL et al., 2014). Consequentemente, este aumento na produção de EROs e ERNs pode levar a reativação das vias inflamatórias em um processo cíclico e crônico (MITTAL et al.,

2014; NGUYEN et al., 2011). Comenta-se também que níveis aumentados de espécies reativas também levam a um aumento da excitabilidade neuronal, especialmente por aumento da sinalização glutamatérgica (CALABRESE et al., 2007; NGUYEN et al., 2011). Por estes motivos, compostos com potencial antioxidante também devem ser levados em conta na pesquisa por drogas que possam ser usadas no tratamento de patologias relacionadas a dor crônica com cunho inflamatório. Estudos já demonstraram efeito positivo (efeito protetor ou coadjuvante) de determinados compostos antioxidantes de origem natural ou sintética em modelos de doenças inflamatórias crônicas e neuropáticas, abrindo caminho para a pesquisa de novas moléculas com atividade semelhante (CHAPPLE, 1997; GRIMBLE, 1994; MOURA et al., 2015).

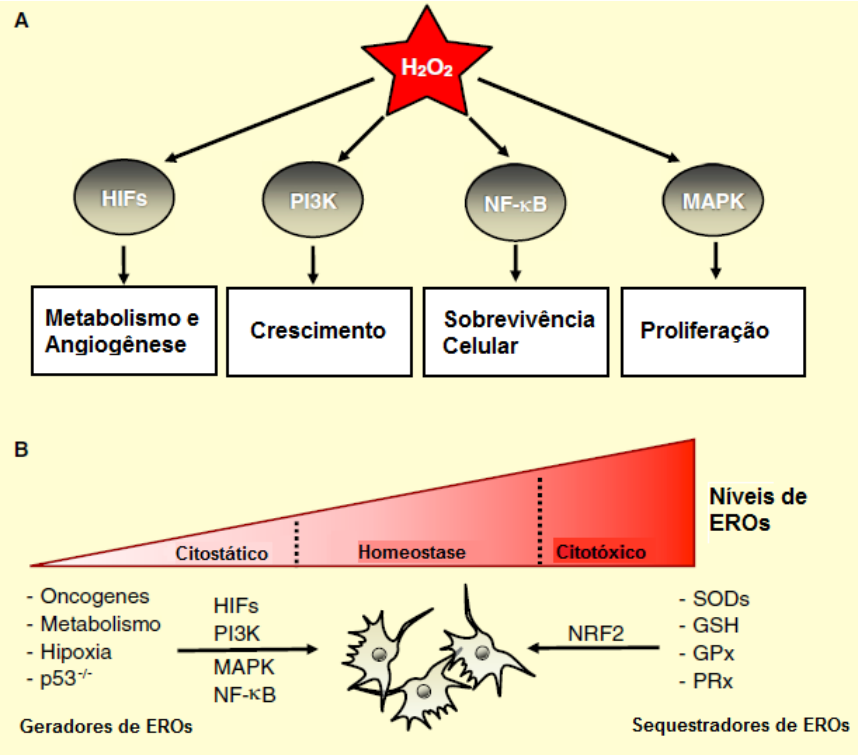

Figura 6. Regulação da proliferação celular e inflamação pelas EROs. (A) Vias celulares ativadas por EROs e (B) alguns mecanismos de aumento ou diminuição dos níveis de EROs. Adaptado de SCHIEBER e CHANDEL, 2014.

1.5 COMPOSTOS ORGÂNICOS DE SELÊNIO E O DISSELENETO DE BIS(FENILIMIDAZOSELENAZOLILA)

Sabe se que o elemento selênio (Se) possui importantes funções fisiológicas, devido ao seu papel em diversas vias metabólicas, dentre as quais podemos citar as funções tireoidiana, imunológica, de síntese de ácido desoxirribonucleico (DNA), e

principalmente no sistema de defesa antioxidante, atuando de forma tanto não-enzimática quanto incorporado a família de selenoenzimas (RAYMAN, 2000).

Além dos compostos orgânicos de selênio naturais (*e. g.*, selenocisteína e selenometionina), compostos sintéticos também têm despertado interesse científico nas últimas décadas devido a suas propriedades bioquímicas e farmacológicas, tendo em vista o seu potencial terapêutico para o uso no tratamento de diversas doenças humanas (NOGUEIRA e ROCHA, 2011; NOGUEIRA et al., 2004a; SORIANO-GARCIA, 2004). Estudos com estes compostos relatam seu potencial como moléculas antioxidantes em modelos estresse oxidativo *in vitro* e *in vivo* (ACKER et al., 2009a; BORTOLATTO et al., 2013; MEOTTI et al., 2004; PRIGOL et al., 2009). Sua atividade antioxidante tem sido relacionada à sua capacidade de mimetizar enzimas como a GPx, GST, deidroascorbato redutase (DHAR), assim como agir como substrato para a tiorredoxina redutase (LUCHESE e NOGUEIRA, 2010a; NOGUEIRA et al., 2004a; SAUSEN DE FREITAS et al., 2010).

Estudos demonstram que um dos protótipos dos compostos orgânicos de selênio, o disseleneto de difenila $(PhSe)_2$ (Figura 8A), assim como seus derivados, parece ter o SNC como um dos principais tecidos alvo, uma vez que apresentam natureza lipofílica, estes são capazes de atravessar facilmente membranas, assim como a barreira hematoencefálica (BHE) (BRÜNING et al., 2014; NOGUEIRA et al., 2004a; PRIGOL et al., 2010). Além disso, a propriedade dos disselenetos de diarila de penetrar a BHE parece ser de extrema importância para seus efeitos farmacológicos, uma vez que já foram reportados suas atividades do tipo-antidepressiva (ACKER et al., 2009b; SAVEGNAGO et al., 2008), ansiolítica (BRUNING et al., 2009b; SAVEGNAGO et al., 2008) e nootrópica (BORTOLATTO et al., 2012; SOUZA et al., 2010).

Devido ao papel do sistema antioxidante em patologias envolvendo dor e inflamação, estudos também foram realizados visando a pesquisa das atividades antinociceptivas e anti-inflamatórias dos compostos orgânicos de selênio. O $(PhSe)_2$, por exemplo, exerce atividade antinociceptiva em modelos de comportamento de lamber a pata induzido por glutamato, formalina e capsaicina e nocicepção visceral induzida por ácido acético e esses efeitos parecem estar relacionados aos sistemas serotoninérgico, glutamatérgico e via do óxido nítrico, mas não a mecanismos opioides (NOGUEIRA et al., 2003; ZASSO et al., 2005). Estudos também relatam atividades similares apresentadas por outros disselenetos de diarila substituídos,

como o disseleneto de *p*-metoxi-difenila $(MeOPhSe)_2$ e o Disseleneto de *m*-trifluorometil-difenila $(F_3CPhSe)_2$, os quais possuem efeito antinociceptivo por mecanismos GABAérgicos e opioides, respectivamente (BRUNING et al., 2010; PINTO et al., 2008). Estes compostos também foram estudados frente a modelos de dor inflamatória ou neuropática, assim como pleurisia induzida por carragenina, modelos nos quais apresentaram efeitos promissores (BRUNING et al., 2015; LUCHESE et al., 2012; SAVEGNAGO et al., 2007a).

Considerando que pequenas mudanças na estrutura de moléculas podem modificar de forma completa ou parcial o efeito de uma droga, novas moléculas baseadas na estrutura do $(PhSe)_2$ foram sintetizadas. Como exemplo, o composto disseleneto de bis(fenilimidazoselenazolila) (DFIS; Figura 8B) é um novo derivado dos disselenetos de diarila. Quanto as suas características estruturais, além de apresentar um grupamento diseleneto, este composto exibe também a presença de dois outros grupamento contendo selênio em sua composição, na forma de grupamentos do tipo selenazol (ROEHRS et al., 2012). Estudos demonstraram que o moléculas contendo o grupamento selenazol, assim como os diselenetos, também apresentam atividade anti-inflamatória, avaliada frente a inflamação induzida por lipopolissacarídeo em cultura de células, e isto deve-se a seu efeito inibitório sobre a produção de óxido nítrico e a ativação da via do NFκB (NAM et al., 2008; PARK et al., 2003). Com base nas propriedades antinociceptivas e anti-inflamatórias já descritas dos compostos orgânicos de selênio antioxidantes, assim como as características estruturais do DFIS, a avaliação dos efeitos deste novo composto em modelos de nocicepção e inflamação, tanto agudos quanto crônicos, é um importante ponto a ser investigado.

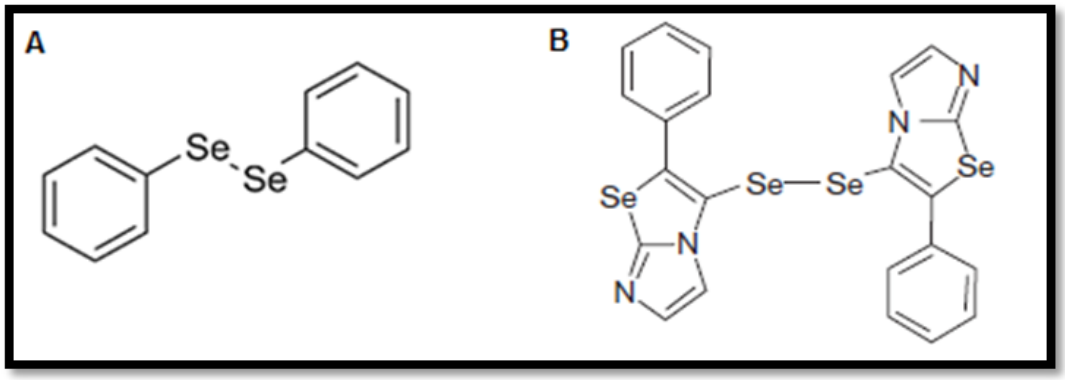

Figura 7 – Estrutura dos compostos (A) $(PhSe)_2$ e (B) DFIS.

2 OBJETIVOS

2.1 Objetivos gerais

Esta tese teve como objetivo investigar os efeitos do disseleneto de bis(fenilimidazoselenazolila) em modelos de nocicepção induzida por diferentes agentes, assim como estabelecer se há uma relação entre o efeito antioxidante do composto e sua ação antinociceptiva.

2.2 Objetivos específicos

Considerando os aspectos mencionados, os objetivos específicos desta tese compreendem:

- Avaliar o possível efeito antinociceptivo do DFIS em modelos de nocicepção aguda induzida por agentes térmicos e químicos;
- Determinar seu potencial antioxidante;
- Verificar a possível toxicidade do DFIS;
- Investigar o efeito do DFIS frente ao modelo de nocicepção inflamatória induzida por adjuvante completo de Freund (CFA);
- Averiguar o efeito do DFIS frente ao modelo de artrite reumatoide induzida por colágeno (AIC).

3 DESENVOLVIMENTO

O desenvolvimento desta tese está apresentado sob a forma de dois artigos científicos e dois manuscritos. Os itens Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se no próprios artigos ou manuscritos, os quais estão estruturados de acordo com as normas de cada revista onde foram publicados ou submetidos, respectivamente.

Em anexo a esta tese encontram-se as autorizações da editora para reprodução dos artigos científicos, bem como a aprovação do projeto de pesquisa pela Comissão de Ética no Uso de Animais (CEUA) da Universidade Federal de Santa Maria.

3.1 Artigo 1

Disseleneto de Bis(fenilimidazoselenazolila): Um Composto com Propriedades Antinociceptivas em Camundongos

BIS(PHENYLIMIDAZOSELENAZOLYL) DISELENIDE: A COMPOUND WITH ANTINOCICEPTIVE PROPERTIES IN MICE

Pietro Maria Chagas, Cristiani Folharini Bortolatto, Ethel Antunes Wilhelm, Juliano Alex Roehrs, Cristina Wayne Nogueira

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Bis(phenylimidazoselenazolyl) diselenide: a compound with antinociceptive properties in mice

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The present study examined the effect of peroral administration of bis(phenylimidazoselenazolyl) diselenide (BPIS) in thermal and chemical models of pain in mice. The involvement of the opioid system in the BPIS antinociceptive effect was also examined, as well as potential nonspecific disturbances in locomotor activity or signs of acute toxicity. BPIS (25–100 mg/kg) induced an increase in tail-immersion response latency and this effect was significant at pretreatment times of 15 min to 4 h, but not at 8 h. The hot-plate response latency was also increased by the administration of BPIS (25–100 mg/kg). BPIS, at doses of 25 and 50 mg/kg, inhibited writhing behaviour caused by an intraperitoneal acetic acid injection. Both early and late phases of nociception caused by the intraperitoneal formalin injection were inhibited by BPIS (10–50 mg/kg). BPIS, administered at doses equal to or greater than 10 and 25 mg/kg, reduced nociception produced by an intraperitoneal injection of capsaicin and glutamate, respectively. The antinociceptive effect of BPIS, when assessed in the tail-immersion test, was not abolished by naloxone. BPIS (10–50 mg/kg) did not alter alanine transaminase and aspartate transaminase activities (parameters of hepatic function) or urea and creatinine levels (parameters of renal function), and did not affect motor activity in the open-field test. The results indicate that BPIS produced an antinociceptive action without causing motor disturbances or toxicity. Moreover, opioidergic mechanisms seem not to be involved in the antinociceptive action of BPIS. Here, BPIS has been found to be a novel organoselenium compound with antinociceptive properties; however, more studies are required to examine its therapeutic potential for pain treatment. *Behavioural Pharmacology* 24:37–44 © 2013 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Keywords: diselenide, mice, nociception, organoselenium, toxicity

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Introduction

Unrelieved acute or chronic pain is often related to negative consequences in various aspects of patients' health-related quality of life (Miller and Cano, 2009; Sinatra, 2010). The analgesic drugs currently available for pain relief, albeit efficacious, still raise several concerns in terms of their safety and side-effects (Steinmeyer, 2000; Jage, 2005). Hence, there is a significant need for exploring more promising and well-tolerated analgesic drugs.

Convincing evidence now indicates that the endogenous antioxidant system plays an important role in the regulation of pain pathways (Rossato *et al.*, 2010). Moreover, pathologies related to pain and inflammation processes, such as fibromyalgia and rheumatoid arthritis, are also believed to involve free radicals and oxidative stress generation (Arranz *et al.*, 2010; Wruck *et al.*, 2011). Some natural and synthetic antioxidants are reported to have antinociceptive activities, including organoselenium compounds, which show anti-inflammatory, antinociceptive and antiallodynic effects in different animal models of nociception (Shin *et al.*, 2009; Valério *et al.*, 2009; Wilhelm *et al.*, 2009).

In this context, antioxidants diaryl diselenides and their derivatives deserve special attention. Diphenyl diselenide $(PhSe)_2$ exerts an antinociceptive action when assessed in models of licking behaviour induced by an intraperitoneal injection of glutamate, formalin or capsaicin, and visceral pain induced by acetic acid, and these effects are not influenced by opioidergic mechanisms, but rather seem to be related to serotonergic, nitrergic and glutamatergic pathways (Nogueira *et al.*, 2003; Zasso *et al.*, 2005; Savegnago *et al.*, 2007). There is evidence supporting the idea that *p*-methoxy-diphenyl diselenide $(MeOPhSe)_2$ and *m*-trifluoromethyl-diphenyl diselenide $(F_3CPhSe)_2$, disubstituted diaryl diselenides, produce antinociception similar to that of $(PhSe)_2$, and these effects involve GABAergic (Pinto *et al.*, 2008) and opioidergic mechanisms (Brüning *et al.*, 2010), respectively.

Considering that slight changes in molecular structures could partially or completely modify the effect of a drug, novel molecules based on the structure of diaryl diselenides have been synthesized. The compound bis(phenylimidazoselenazolyl) diselenide (BPIS) is a novel diaryl diselenide derivative and it has been shown to be

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a promising antioxidant *in vitro* (P.M. Chagas, E.A. Wilhelm, J.A. Roehrs, C.W. Nogueira, unpublished data) compared with other diselenides. Therefore, on the basis of the antinociceptive property already described for antioxidant diaryl diselenides, the aims of the present study were to examine (a) the antinociceptive action of BPIS in different models of acute nociception; (b) the possible involvement of the opioid system in the antinociceptive action of BPIS; (c) possible nonspecific disturbances in the locomotor activity of mice treated with BPIS; and (d) the potential acute toxicity caused by BPIS in mice.

Methods

Subjects

The experiments were conducted using male adult Swiss mice (25–35 g) from our own breeding colony. The animals were kept in a separate animal room, on a 12-h light/dark cycle with lights on at 07:00 h, at room temperature (22±1°C) with free access to water and food. All manipulations were carried out between 08:00 and 16:00 h. All experiments were conducted on separate groups of animals and each animal was used only once and only in a single test to avoid interference between tests. Experiments were conducted according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, the Federal University of Santa Maria, Brazil. All efforts were made to minimize suffering and to reduce the number of animals used in the experiments. Animals were divided randomly into groups of seven to nine animals each.

Thermal models of nociception

Tail-immersion test

The tail-immersion test was carried out as described by Janssen *et al.* (1963). The test was performed by immersing the lower 3.5 cm of the tail into a cup freshly filled with water from a large constant-temperature (55°C) bath until the typical tail withdrawal response was observed. A 7 s cut-off was imposed. Changes in tail-immersion latency, Δt (s), were calculated for each animal according to the formula [Δt (s) = postdrug latency – predrug latency] (Pinardi *et al.*, 2003).

To assess the time course of the antinociceptive effect of BPIS, mice were pretreated with BPIS (50 mg/kg, orally) or vehicle (canola oil, 10 ml/kg, orally) at 15, 30 min, 1, 2, 4 and 8 h before the test; the control group received canola oil 30 min before the test. To examine the dose–response effect of BPIS, the compound was administered (10–100 mg/kg, orally) 30 min before the test.

Morphine (2.5 mg/kg, subcutaneously), administered 30 min before the test, was used as a positive control (Brüning *et al.*, 2010).

Hot-plate test

The hot-plate test was carried out according to the method described previously (Woolfre and MacDonald, 1944). In this experiment, the hot-plate apparatus was maintained at 55±0.1°C. Animals were placed in an acrylic cylinder (20 cm in diameter) on the heated surface, and the time (s) between placement and licking of their hind paws or jumping was recorded as the response latency. A 60 s cut-off was used to prevent tissue damage. Twenty-four hours before the experiment, all mice were habituated to the experimental procedure to minimize novelty-induced antinociception (Siegfried *et al.*, 1987). Animals presenting training latencies higher than 20 s were excluded. On the day of the experiment, animals were treated with BPIS (10–100 mg/kg, orally) or vehicle and subjected to the hot-plate test 30 min thereafter. Morphine (2.5 mg/kg, subcutaneously), administered 30 min before the test, was used as a positive control (Khazaeli *et al.*, 2010).

Chemical models of nociception

Acetic acid-induced abdominal writhing

Abdominal constrictions were induced by acetic acid (1.6%, intraperitoneally) according to the procedure described previously (Corrêa *et al.*, 1996). After the injection of acetic acid, mice were individually placed in separate boxes and the abdominal constrictions were counted cumulatively over a period of 20 min. Mice were pretreated with BPIS (10–50 mg/kg, orally) 30 min before the injection of acetic acid. Control animals received a similar volume of vehicle, and as a positive control, morphine (2.5 mg/kg, subcutaneously) was administered 30 min before the test (Savegnago *et al.*, 2007).

Formalin test

The formalin test was carried out as described previously (Hunskaar and Hole, 1987; Okuda *et al.*, 2001). Animals received an intraperitoneal administration of formalin (2.5%, v/v; 20 µl/paw) into the ventral surface of the right hind paw. After formalin injection, mice were returned to the experimental cage and the time spent licking the injected paw was recorded during the periods of 0–5 min (early neurogenic phase) and 15–30 min (late inflammatory phase). BPIS (10–50 mg/kg, orally) or vehicle were administered 30 min before the injection of formalin into the ventral right (ipsilateral) hind paw. Morphine (2.5 mg/kg, subcutaneously) (Khazaeli *et al.*, 2010) or diclofenac sodium (10, 20 and 40 mg/kg, intraperitoneally) (Cristiano *et al.*, 2008), administered 30 min before the test, were used as a positive control.

Glutamate-induced nociception

The procedure used was similar to that described previously (Meotti *et al.*, 2010). Mice were treated with BPIS (10–50 mg/kg, orally) or vehicle 30 min before an intraperitoneal injection of glutamate (20 µmol, 20 µl/paw) on the ventral surface of the right hind paw. Mice were observed individually for 15 min following an injection of glutamate and the amount of time spent licking the

injected paw was recorded using a chronometer. Morphine (2.5 mg/kg, subcutaneously), administered 30 min before the test, was used as a positive control (Freitas *et al.*, 2009).

Capsaicin-induced nociception

The procedure used was similar to that described previously (Santos *et al.*, 1999). Capsaicin (1.6 µg, 20 µl/paw) was injected into the ventral surface of the right hindpaw. Animals were observed individually for 5 min following capsaicin injection. The amount of time spent licking the injected paw was recorded using a chronometer. Animals were treated with BPIS (1–50 mg/kg, orally) or vehicle 30 min before capsaicin injection.

Role of the opioid system in the antinociceptive effect of bis(phenylimidazoselenazolyl) diselenide in the tail-immersion test

To determine the role of the opioid system in the antinociceptive effect of BPIS, the tail-immersion test was chosen. Mice were pretreated with naloxone, a nonselective opioid antagonist (1 or 10 mg/kg, subcutaneously), or saline (vehicle) (Savegnago *et al.*, 2007) 15 min before BPIS (50 mg/kg, orally), morphine (2.5 mg/kg, subcutaneously, as a positive control) or their respective vehicles. The tail-immersion test was carried out 30 min later.

Open-field test

Spontaneous exploratory behaviour was assessed in the open-field test to rule out any motor disturbance related to the administration of BPIS. The open field was made of plywood and surrounded by walls 30 cm in height. The floor of the open field, 45 cm in length and 45 cm in width, was divided by masking tape markers into nine squares (three rows of three). Animals were evaluated 30 min after a single oral dose of vehicle or BPIS (10–50 mg/kg). Each animal was placed individually at the centre of the apparatus and observed for 6 min to record the number of segments crossed with the forepaws and the number of rears on the hind limbs (Walsh and Cummins, 1976).

Acute toxicity

To examine the potential acute toxicity caused by BPIS, mice received a single oral dose of BPIS (10–50 mg/kg) or vehicle. After drug administration, animals were observed up to 72 h to determine the lethal potential of BPIS. The animals were separated four to five per cage and all mice in the same cage received the same treatment ($n = 8–9$). The individual gain in body weight was recorded and calculated according to the formula: [baseline body weight (obtained before the beginning of treatment) – body weight at the end of the experiment]. Water and food consumption was measured daily in mice exposed to BPIS. The average weight of water and food consumed was calculated according to the formula: [water and food intake (g)/number of animal per cage].

After 72 h, mice were anaesthetized and blood was collected by heart puncture in tubes containing heparin. Plasma was obtained by centrifugation at 2000*g* for 10 min (haemolysed plasma was discarded) and used for biochemical assays, which were performed using commercial test kits. Plasma aspartate aminotransferase and alanine aminotransferase activities, used as the biochemical markers for early acute hepatic damage, were determined using the colorimetric method of Reitman and Frankel (1957). Renal function was analysed by determining plasma urea (Makay and Mackay, 1927) and creatinine levels (Jaffe, 1886).

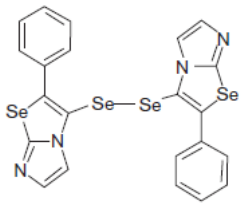
Drugs

BPIS (Fig. 1) was prepared and characterized according to Roehrs *et al.* (2012). Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed analytical and spectroscopic data in full agreement with its assigned structure (Roehrs *et al.*, 2012). The chemical purity of BPIS (99.9%) was determined by GC/MS. Capsaicin and naloxone hydrochloride were purchased from the Sigma Chemical Co. (St Louis, Missouri, USA). All other chemicals were of analytical grade and obtained from standard commercial suppliers. All drugs were dissolved in saline, except BPIS, which was dissolved in canola oil. The mice received drugs by the oral (administered by an intragastric gavage), subcutaneous and intraperitoneal routes at a constant volume of 10 ml/kg body weight. Appropriate vehicle-treated groups were also assessed simultaneously.

Statistical analysis

The results are presented as mean ± SEM. Differences between groups were analysed by means of one-way (tail-immersion, hot-plate, acetic acid-induced abdominal writhing, formalin, glutamate, capsaicin, and open-field tests, and parameters of acute toxicity) or two-way (investigation of the possible involvement of the opioid system in the antinociceptive effect of BPIS) analysis of variance, followed by the Newman–Keuls test when appropriate. The criterion for statistical significance was P value less than 0.05, which was considered as statistically significant. Effective dose 50 (ED_{50}) values (i.e. the dose of BPIS that reduced the pain response by

Fig. 1



Chemical structure of bis(phenylimidazoselenazolyl) diselenide.

50% in relation to control group values or the dose with the maximal effect in the thermal tests) were determined by linear regression GraphPad Software (GraphPad Software Inc., San Diego, California, USA), and are reported as the median effective dose accompanied by their respective 95% confidence limits.

Results

Thermal models of nociception

Tail-immersion-induced nociception

The antinociceptive effect of BPIS, at a dose of 50 mg/kg, reached its peak 30 min after oral administration and remained significant up to 4 h [$F(6,50) = 6.03$, $P < 0.001$] (Fig. 2a). Thus, the time point of the maximum effect (30 min) was chosen for all further studies.

BPIS, at doses of 25, 50 and 100 mg/kg, and the positive control morphine, administered 30 min earlier, led to a significant increase in the tail-immersion response latency relative to the control group [$F(5,41) = 7.90$; $P < 0.001$] (Fig. 2b). The ED_{50} for BPIS was 14.49 (6.48–32.41) mg/kg. No significant effect of BPIS was observed at the lowest dose (10 mg/kg).

The mean baseline latencies were 2.13±0.12 s [$F(6,50) = 1.09$; NS] in the time-course study and 1.54±0.10 s [$F(5,41) = 1.07$; NS] in the dose–response study, and were not significantly different between groups.

Hot-plate test

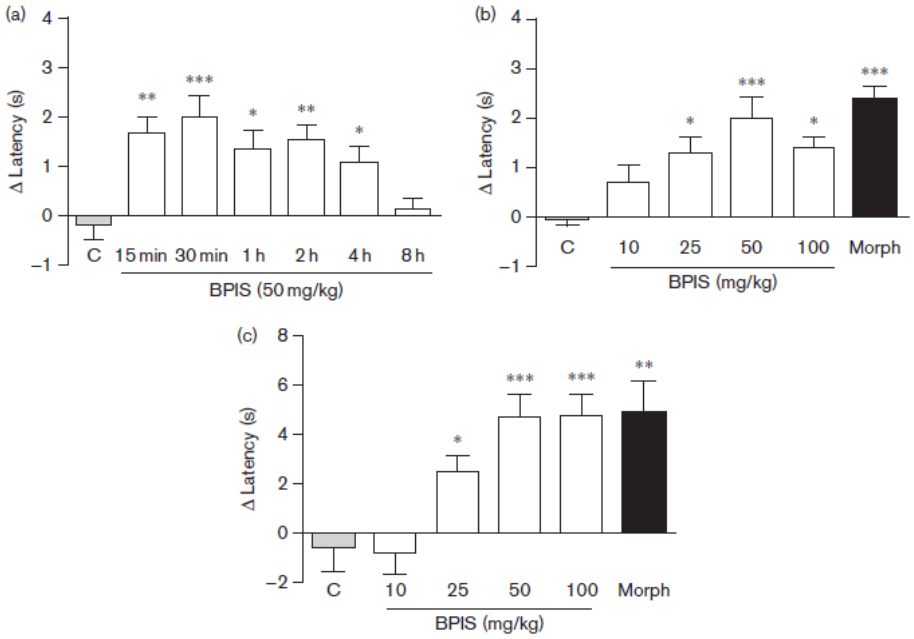
In the hot-plate test, treatment with BPIS, at doses of 25, 50 and 100 mg/kg, or morphine, increased response latency to thermal stimuli, compared with the control group [$F(5,41) = 8.80$; $P < 0.001$] (Fig. 2c). No significant effect was observed at the lowest dose of BPIS (10 mg/kg). The ED_{50} was 26.97 (19.93–36.49) mg/kg. A dose of 100 mg/kg did not cause any further increase in the antinociceptive effect observed at 50 mg/kg BPIS. As no dose-dependent effect was found, the 100 mg/kg dose was not used in subsequent experiments. The mean baseline latency was 5.86±0.21 s [$F(5,41) = 0.25$; NS], and did not differ significantly between groups.

Chemical models of nociception

Acetic acid-induced abdominal writhing

As shown in Fig. 3a, BPIS, at doses of 25 and 50 mg/kg, but not at 10 mg/kg, caused a significant decrease in the

Fig. 2



Effect of bis(phenylimidazoselenazolyl) diselenide (BPIS) in mouse thermal nociception tests. (a) Time course of the response to BPIS in the tail-immersion test; the control group received canola oil only 30 min before the test; (b) dose–response curve for BPIS administered 30 min before the tail-immersion test; (c) Effect of BPIS in the hot-plate test. Each bar represents the mean±SEM of seven to nine mice in each group. [C] indicates animals treated with canola oil and [Morph] animals treated with the control-positive morphine. *$P<0.05$; **$P<0.01$; ***$P<0.001$; one-way analysis of variance, followed by the Newman–Keuls test.

number of writhes [$F(4,33) = 29.75$; $P < 0.001$]. The ED_{50} was 37.26 (31.77–43.71) mg/kg.

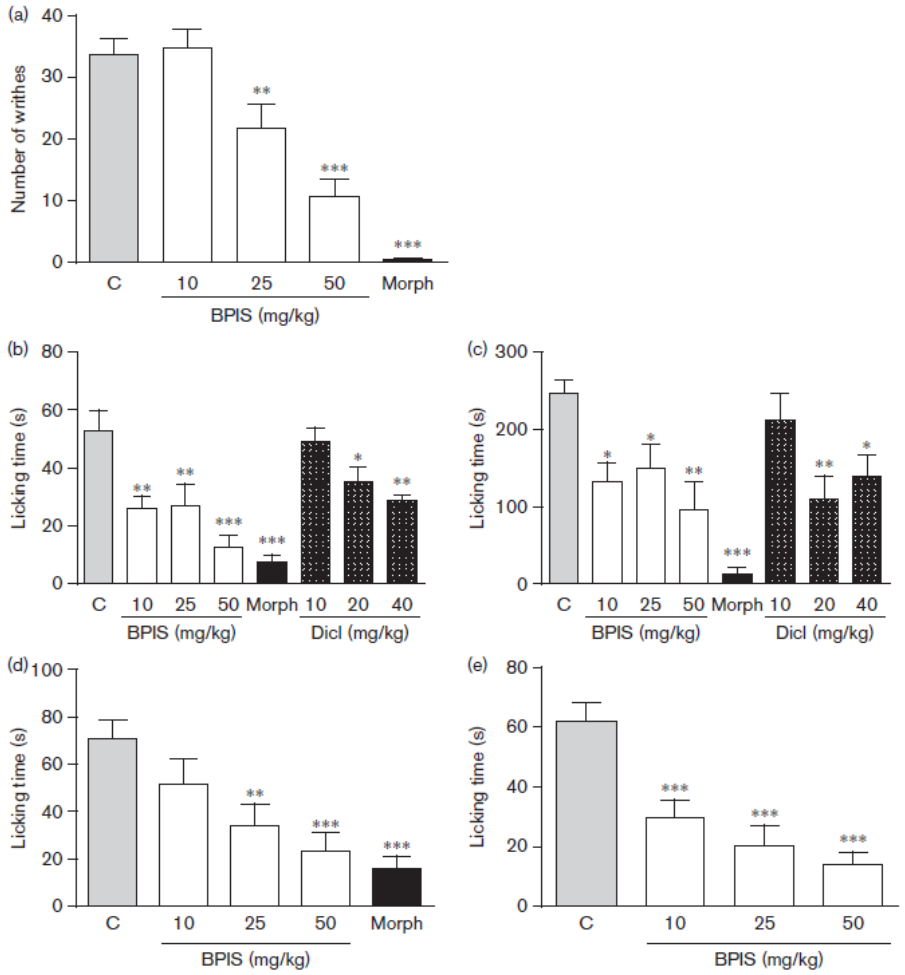
Formalin test

The effect of BPIS on the time spent licking the formalin-injected paw during the first (0–5 min) and second phases (15–30 min) of the test is shown in Fig. 3b and c, respectively. BPIS, at doses of 10 mg/kg or higher, decreased the time spent licking the hind paw in the first [$F(7,52) = 9.67$; $P < 0.001$] and second phases [$F(7,52) = 7.29$; $P < 0.001$] of the formalin test. Morphine (2.5 mg/kg; subcutaneously) and diclofenac at doses of 20 and 40 mg/kg, but not at 10 mg/kg (intraperitoneally), also decreased the time spent licking the hind paw, in both phases. The ED_{50} values for BPIS were 25.60 (17.42–37.61) mg/kg for the first phase and 34.23 (27.94–41.93) mg/kg for the second phase.

Glutamate-induced nociception

Figure 3d shows that treatment with BPIS (at 25 and 50 mg/kg, but not at 10 mg/kg) resulted in a significant inhibition of glutamate-induced nociception [$F(4,40) = 8.77$; $P < 0.001$]. The ED_{50} was 27.13 (16.77–43.89) mg/kg.

Fig. 3



Effect of bis(phenylimidazoselenazolyl) diselenide (BPIS) in chemical nociception tests in mice. (a) Acetic acid-induced writhing test; (b) first (0–5 min) phase of the formalin test; (c) second (15–30 min) phase of the formalin test; (d) licking behaviour induced by glutamate; (e) licking behaviour induced by capsaicin. Each bar represents the mean±SEM of seven to nine mice in each group. [C] indicates animals treated with canola oil; [Morph] and [Dicl] indicate animals treated with the positive controls morphine and diclofenac, respectively. *$P<0.05$; **$P<0.01$; ***$P<0.001$; one-way analysis of variance, followed by the Newman–Keuls test.

Capsaicin-induced nociception

BPIS, at all tested doses, caused a significant reduction in the capsaicin-induced licking response [$F(3,28) = 13.50$; $P < 0.001$] (Fig. 3e). The ED_{50} was 18.62 (10.45–33.16) mg/kg.

The role of the opioid system in the antinociceptive effect of bis(phenylimidazoselenazolyl) diselenide in the tail-immersion test

Pretreatment with naloxone at a dose of 1 mg/kg (subcutaneously) abolished the antinociceptive effect of morphine (positive control) at a dose of 2.5 mg/kg (subcutaneously) [$F(1,24) = 12.71$; $P < 0.001$]. By contrast, the antinociceptive effect of BPIS (50 mg/kg, orally) was not blocked by pretreatment with naloxone at doses of 1 mg/kg [$F(1,25) = 3.33$; NS] or 10 mg/kg [$F(1,26) = 0.07$; NS] (Fig. 4). The mean baseline latency was 1.78±0.07 s [$F(7,50) = 0.32$; NS] and did not differ significantly between groups.

Open-field test

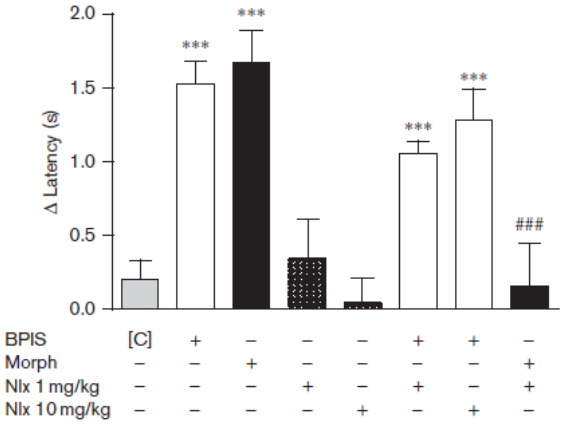
Treatment of mice with BPIS (10–50 mg/kg, orally) did not cause any significant change in the number of crossings [$F(3,28) = 0.2450$; NS] or rears [$F(3,28) = 0.17$; NS] in the open-field test (Table 1).

Acute toxicity

A single oral administration of BPIS (10–50 mg/kg) reduced neither body weight gain [$F(3,32) = 1.90$; NS] nor food intake [$F(3,32) = 0.17$; NS] (Table 2), and did not cause any fatalities. Only water intake was reduced in animals exposed to BPIS at a dose of 50 mg/kg [$F(3,32) = 9.03$; $P < 0.001$] (Table 2).

The activities of plasma alanine aminotransferase [$F(3,32) = 0.85$; NS] and aspartate aminotransferase [$F(3,32) = 0.47$; NS] were unchanged by BPIS treatment, relative to the control group (Table 3). There was also no change in the levels of urea [$F(3,32) = 1.09$; NS] and creatinine [$F(3,32) = 0.82$; NS] in animals treated with BPIS (Table 3).

Fig. 4



Role of the opioid system in the antinociceptive effect of bis(phenylimidazoselenazolyl) diselenide (BPIS) in the tail-immersion test. [C] indicates animals treated with canola oil, [BPIS] animals treated with BPIS (50 mg/kg), [Morph] animals treated with the positive control morphine and [Nlx] animals treated with naloxone. Each bar represents the mean±SEM of seven to nine animals in each group. ***P<0.001 compared with the control group; ###P<0.001 compared with the morphine group; two-way analysis of variance, followed by the Newman–Keuls test.

Discussion

In the present study, we found that BPIS elicited an antinociceptive action in chemical and thermal models of pain in mice, and that this action seems not to be related to opioidergic mechanisms. The results also indicate that the administration of BPIS to mice caused neither acute toxicity nor nonspecific locomotor disturbances.

Administration of BPIS to mice caused a significant prolongation of response latency in the tail-immersion and hot-plate tests, indicating an increase in the nociceptive threshold similar to that caused by morphine, the positive control (administered at a different dose and by a different route). These results indicate that BPIS was effective in inhibiting thermal nociception at different levels of the central nervous system, as the hot-plate test mainly reflects supraspinal responses and the tail-immersion test reflects spinal responses (Langerman *et al.*, 1995).

Table 1 Effect of oral administration of bis(phenylimidazoselenazolyl) diselenide in the mouse open-field test

Groups	Number of crossings	Number of rears
Control	81.63±7.66	38.63±3.98
10 mg/kg	74.38±9.02	38.00±4.08
25 mg/kg	82.38±4.58	43.38±4.25
50 mg/kg	75.88±10.19	39.88±9.20

Animals were pretreated with bis(phenylimidazoselenazolyl) diselenide (BPIS), at doses from 10 to 50 mg/kg, 30 min before the open-field test. Data are reported as the mean±SEM of seven to nine mice in each group.

Table 2 Effect of oral administration of bis(phenylimidazoselenazolyl) diselenide on body weight gain, food intake and water intake in mice

Groups	Weight gain (g/animal)	Food intake (g/animal)	Water intake (g/animal)
Control	−0.11±0.45	30.78±3.17	21.44±0.10
10 mg/kg	0.56±0.38	29.33±0.69	21.00±0.31
25 mg/kg	0.67±0.52	27.44±1.99	20.89±1.06
50 mg/kg	1.33±0.33	33.56±0.77	17.33±0.61***

Animals were pretreated with bis(phenylimidazoselenazolyl) diselenide (BPIS), at doses from 10 to 50 mg/kg, 72 h before determinations. Data are reported as the mean±SEM of seven to nine mice in each group. ***P<0.001; one-way analysis of variance followed by the Newman–Keuls test.

Table 3 Effect of oral administration of bis(phenylimidazoselenazolyl) diselenide on plasma biochemical parameters in mice

Groups	ALT (U/L)	AST (U/L)	Urea (mg/dl)	Creatinine (mg/dl)
Control	55.00±3.06	70.00±3.03	47.59±3.18	0.442±0.023
10 mg/kg	50.89±3.71	67.53±4.71	54.78±4.25	0.471±0.030
25 mg/kg	54.67±3.75	75.46±3.63	45.69±4.01	0.424±0.030
50 mg/kg	42.43±2.88	70.64±5.14	50.37±3.64	0.490±0.033

Animals were pretreated with bis(phenylimidazoselenazolyl) diselenide (BPIS), at doses from 10 to 50 mg/kg, 72 h before determinations. Data are reported as the mean±SEM of seven to nine mice in each group.
ALT, alanine aminotransferase; AST, aspartate aminotransferase.

The time-course study in the tail-immersion test yielded the interesting finding that the antinociceptive effect of BPIS remained significant for up to 4 h after administration. Studies have shown that the effects of other organoselenium compounds, such as $(PhSe)_2$ and bis-selenide derivatives, remain significant up to 2 h and 45 min, respectively (Savegnago *et al.*, 2006; 2007), and that effects of morphine, diacetylmorphine and 6-acetylmorphine, known analgesic drugs, last 2.5, 1.8 and 1.7 h, respectively (Umans and Inturrisi, 1981).

The present data indicated that BPIS inhibited the acetic acid-induced visceral nociceptive response in mice. Although the acetic acid-induced writhing is a test weakly predictive of antinociceptive activity because of its lack of specificity, showing false-positive responses to some drugs that have no analgesic action (Le Bars *et al.*, 2001), this result indicates a potential antinociceptive action of BPIS in a chemical model of nociception.

In the formalin test, BPIS also inhibited both the first, or neurogenic, phase, which results basically from the direct activation of nociceptors, and the second phase, which involves a period of sensitization during which an inflammatory phenomenon occurs (McNamara *et al.*, 2007). The effect of BPIS in this test is an important finding, because it indicates an antinociceptive effect of this compound against inflammatory pain. Also, this model is considered one of the most predictive of drugs used to treat acute pain (Le Bars *et al.*, 2001).

On the basis of these antinociceptive effects of BPIS in different screening tests for analgesic drugs, we sought to determine whether BPIS inhibits the nociceptive response caused by an intraperitoneal injection of glutamate. BPIS was indeed effective in inhibiting glutamate-induced nociceptive behaviour. The neurotransmitter glutamate plays important roles in nociception: it is stored principally in C-fibres, released in the spinal cord or in peripheral nerves in response to noxious stimuli or inflammation, and activated ionotropic and/or metabotropic glutamatergic receptors (Bleakman *et al.*, 2006). Studies have shown that the antinociceptive effect of some diaryl diselenides, such as $(MeOPhSe)_2$ and $(PhSe)_2$, involves glutamatergic mechanisms (Savegnago *et al.*, 2007; Pinto *et al.*, 2008).

BPIS was also effective in inhibiting the nociceptive behaviour induced by an intraperitoneal injection of capsaicin, a selective agonist of the vanilloid TRPV1 receptor. This receptor is activated by different chemical (capsaicin and acid) or physical (heat) stimuli and its activity can be heightened by agents involved in inflammation processes, such as nerve growth factor and bradykinin (Julius and Basbaum, 2001). The administration of capsaicin and consequent activation of TRPV1 receptors has been shown to lead to glutamate release in primary afferent fibres and to potentiate the glutamate input to areas such as the dorsolateral periaqueductal grey, an important area in pain modulation (Xing and Li, 2007; Jin *et al.*, 2009). Although these results suggest that vanilloid and glutamatergic systems might be involved in the mechanism of BPIS-induced antinociception, we cannot confirm this hypothesis because chemical irritant pain models have a wide range of nociceptive mediators.

The opioid system is one of the most important systems involved in pain modulation. Drugs that activate different opioid receptors, such as morphine and its derivatives, have notable antinociceptive activity in different models of nociception (Jage, 2005). The results reported here show that the blockade of opioid receptors by the opioid antagonist naloxone was ineffective in antagonizing antinociception elicited by BPIS, suggesting that this effect is not directly related to an interaction with opioid receptors. Accordingly, studies from our research group have shown that in general, organoselenium compounds with antinociceptive action, other than $(F_3CPhSe)_2$, do not interact with the opioid system (Savegnago *et al.*, 2006, 2007; Wilhelm *et al.*, 2009; Brüning *et al.*, 2010).

Changes in motor activity can interfere with the nociceptive response (Le Bars *et al.*, 2001). No alteration in spontaneous exploratory behaviour was found in mice treated with BPIS, ruling out the possibility that locomotor changes caused a false-positive response in the nociceptive models.

To exclude a possible toxicity of BPIS, we examined the potential acute toxicity after oral administration of BPIS to mice. BPIS did not alter body weight gain, food consumption or biochemical markers of renal and hepatic damage, suggesting that there was no acute toxicity. Water consumption was the only toxicity parameter that was slightly reduced in mice treated with BPIS. In this context, results from our research group have shown that organoselenium compounds have an anorectic property, reducing water and food consumption in different species (Meotti *et al.*, 2008; Savegnago *et al.*, 2009).

Together, the present results indicate that BPIS might be of potential significance for the development of new clinically relevant drugs for the treatment of pain. Additional studies are, however, necessary to substantiate this proposition, especially evaluating BPIS in models of

chronic afflictions related to pain, inflammation and oxidative stress, such as rheumatoid arthritis, which result in disability and impairment in the quality of life. The exact molecular mechanism by which BPIS exerts an antinociceptive action also requires investigation.

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Conflicts of interest

There are no conflicts of interest.

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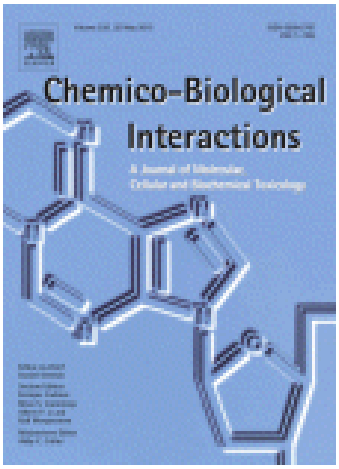
3.2 Artigo 2

Disseleneto de Bis(fenilimidazoselenazolila) como um Composto Antioxidante: Um Estudo *in vitro* e *in vivo*

BIS(PHENYLIMIDAZOSELENAZOLYL) DISELENIDE AS AN ANTIOXIDANT COMPOUND: AN *IN VITRO* AND *IN VIVO* STUDY

Pietro Maria Chagas, Bruna da Cruz Weber Fulco, Ana Paula Pesarico, Juliano Alex Roehrs, Cristina Wayne Nogueira



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Bis(phenylimidazoselenazolyl) diselenide as an antioxidant compound: An *in vitro* and *in vivo* study

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ABSTRACT

The organoselenium compounds have been reported for many biological properties, especially as potent antioxidants. The compound bis(phenylimidazoselenazolyl) diselenide (BPIS) is a novel diaryl diselenide derivative, which shows antinociceptive and anti-inflammatory properties in mice, but whose antioxidant activity has not been studied. The present study aimed to investigate the antioxidant and toxicological potential of BPIS in brain of rats *in vitro*, and the effect of BPIS against the oxidative damage induced by sodium nitroprusside (SNP) in mouse brain. BPIS, at low molecular range, reduced lipid peroxidation (LP) and protein carbonyl (PC) content in rat brain homogenates (IC_{50} values of 1.35 and 0.74 μM, respectively). BPIS also presented dehydroascorbate reductase-like and glutathione-S-transferase-like, as well as DPPH and NO-scavenging activities. Related to togicological assays, BPIS inhibited δ-ALA-D and Na^+, K^+-ATPase activities in rat brain homogenates and [^{3}H]glutamate uptake in synaptosomes *in vitro*, but these effects were observed at higher concentrations than it had antioxidant effect (IC_{50} values of 16.41, 26.44 and 3.29 μM, respectively). *In vivo*, brains of mice treated with SNP (0.335 μmol per site; i.c.v.) showed an increase in LP and PC and a reduction in non protein thiol content, however, it was not observed significant alterations in antioxidant enzyme activities. BPIS (10 mg/kg; p.o.) protected against these alterations caused by SNP. In conclusion, the results demonstrated the antioxidant action of BPIS in *in vitro* assays. Furthermore, BPIS protected against oxidative damage caused by SNP in mouse brain, strengthening the potential antioxidant effect of this compound.

1. Introduction

Reactive oxygen species (ROS), electrophilic molecules which are physiologically produced can react with lipids, proteins and nucleic acids, resulting in oxidative damage to these macromolecules, when produced in high concentrations [4]. ROS can also react with nitric oxide (NO), forming the reactive nitrogen species (RNS), which are equally deleterious [42]. Both species are generally detoxified by cellular enzymatic and non-enzymatic antioxidant compounds, maintaining the natural redox state of the cell [42].

However, in situations when there is an imbalance between the production of ROS/RNS and the natural antioxidants; this event is called oxidative stress, which can lead to cell damage. Studies have reported the involvement of oxidative stress in the pathophysiology of diseases and neurodegenerative processes like aging [4], inflammation [46] and cancer [17].

Sodium Nitroprusside (SNP; Fig. 1A) is considered a NO donor, generally used as an inductor of apoptosis and oxidative stress *in vitro* and *in vivo* [26,23]. SNP interacts with oxyhemoglobin in the blood to produce methemoglobin while releasing cyanide (CN^-) and NO spontaneously [24]. Then, SNP could induce reduction of enzyme mitochondrial activity as well as production of ROS and RNS, triggering to oxidative stress and subsequently cellular damage [5,24].

The interest in the treatment of diseases related to oxidative stress with antioxidants has increased in the last years [31]. Intending to prevent the oxidative stress-related damage, natural and synthetic antioxidants are tested in *in vitro* and *in vivo* models of toxicity [27,20].

In this context, a special attention can be given to the organoselenium compounds and their derivatives. This class of compounds has been reported as potent antioxidants, and this property seems to be related to their ability to mimick enzymes as glutathione peroxidase (GPx), dehydroascorbate reductase (DHAR) and glutathione-S-transferase (GST), as well as act as substrate for the

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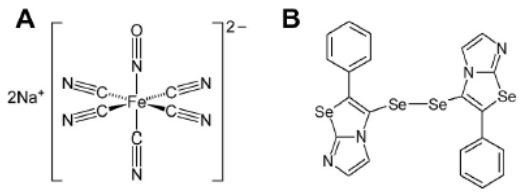

Fig. 1. Chemical structure of (A) SNP and (B) BPIS.

enzyme thioredoxin reductase (TrxR) [32,9]. Considering that slight modifications in molecular structures could partially or completely alter the effect of a drug, novel organoselenium compounds have been synthesized. The compound bis(phenylimidazoselenazolyl) diselenide (BPIS; Fig. 1B) is a novel diaryl diselenide derivative, which has been already proven to have antinociceptive and anti-inflammatory properties in mice [7] but its antioxidant activity has not been studied.

Whereas many of the organoselenium compounds properties are related to their antioxidant activity, the present study aimed to investigate the antioxidant potential of BPIS *in vitro*. Toxicological assays, such as the effect of BPIS on the activity of sulfhydryl enzymes and glutamate uptake, which are end points related to organoselenium compounds toxicity, were also performed. Based on the *in vitro* data, we also evaluated the *in vivo* effect of BPIS on the model of SNP-induced cerebral oxidative stress.

2. Material and methods

2.1. Chemicals

BPIS was prepared and characterized according to Roehrs et al. [40]. Analysis of the ^{1}H NMR and ^{13}C NMR spectra shed analytical and spectroscopic data in full agreement with its assigned structure [40]. The chemical purity of BPIS (99.9%) was determined by GC/MS. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) diammonium salt, δ-aminolevulinic acid (δ-ALA), ascorbic acid, adenosine 5′-triphosphate (ATP) disodium salt hydrate, 1-chloro-2,4-dinitrobenzene (CNDB), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), 2′,7′-dichlorofluorescin diacetate (DCF-DA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), [^{3}H]-L-glutamic acid, glutathione (GSH), SNP, thiobarbituric acid (TBA) and vanadium (III) chloride were purchased from the Sigma Chemical Co. (St Louis, Missouri, USA). All other chemicals were of analytical grade and obtained from standard commercial suppliers. BPIS was dissolved in dimethylsulfoxide (DMSO) and canola oil for *in vitro* and *in vivo* experiments, respectively.

2.2. Animal

Male adult albino Wistar rats (200–300 g) and male adult C57BL/6 mice (25–35 g) from our own breeding colony were used for *in vitro* and *in* vivo experiments, respectively. Animals were kept on a separate animal room, in a 12 h light/dark cycle, at a room temperature of 22 ± 2 °C, with free access to food (Guabi, RS, Brazil) and water. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Brazil (#066/2014).

2.3. Tissue preparation

Animals were killed and the brain tissue was rapidly dissected, weighed and placed on ice. Tissues were immediately homogenized in cold 50 mM Tris–HCl, pH 7.4 (1/5, w/v). Protein carbonyl content was assayed using the fresh brain homogenate without centrifugation. For *in vitro* lipid peroxidation, δ-aminolevulinate dehydratase (δ-ALA-D) and Na^{+}, K^{+}-ATPase assays, the homogenate was centrifuged at 2400*g* for 10 min to yield a pellet that was discarded and a low-speed supernatant (S_1), the latter was used to determine the effect of different concentrations of BPIS on the previously mentioned assays. Differently, for the [^{3}H]glutamate uptake assay, the rats were decapitated and the whole brain was removed and used to prepare synaptosomes [45].

2.4. In vitro experiments

The *in vitro* experiments were carried out in order to investigate the antioxidant and potential toxicological effect of BPIS.

2.4.1. Lipid peroxidation induced by Fe (II)/EDTA

Fe(II)/EDTA were used as classical inductors of lipid peroxidation. An aliquot of 200 μL of S_1 was added to the reaction mixture containing: 30 μL of 500 μM EDTA solution (in water), 30 μL of 1.44 mM $FeCl_2$ solution and 10 μL of BPIS at different concentrations (final concentrations of 0.1 to 5 μM) and water to complete a final volume of 300 μL. The $FeCl_2$ solution was prepared in water, maintained in a dark tube on the ice and immediately used. Afterward the mixture was pre-incubated at 37 °C for 1 h. The reaction product was determined using 500 μL TBA (0.8%), 200 μL sodium dodecyl sulfate (SDS, 8.1%) and 500 μL acetic acid (pH 3.4) with subsequent incubation at 95 °C for 1 h. TBA reactive species (TBARS) were spectrophotometrically determined at 532 nm as previously described [33], using malondialdehyde (MDA, an end product of the peroxidation of lipids) as an external standard. Results were expressed as nmol MDA/g tissue. Ascorbic acid (final concentration 1–1000 μM) was used as positive control.

2.4.2. Protein carbonyl determination

Carbonyl content was assayed by a method based on the reaction of protein carbonyls with dinitrophenylhydrazine (DNPH) forming dinitrophenylhydrazone [39]. Homogenate was diluted with Tris–HCl buffer, pH 7.4 in a proportion of 1:8 (homogenate:Tris–HCl). Aliquots of 940 μL of homogenate dilutions were incubated at 37 °C for 2 h in the presence of 10 μL of BPIS at different concentrations (final concentrations of 0.1–1 μM) and 50 μL of 20 mM SNP. SNP was used to stimulate the protein carbonyl production and was prepared in water, maintained in a dark tube on the ice and immediately used. In two tubes, it was added 200 μL of 10 mM DNPH in 2.0 M HCl. In the third tube, only 200 μL of 2.0 M HCl solution (blank) was added. All tubes were incubated for 1 h at room temperature, in dark and shaken using a vortex mixer every 15 min. After that, 0.5 mL of denaturizing buffer (sodium phosphate buffer, pH 6.8, containing 3% SDS), 1.5 mL of ethanol and 1.5 mL of hexane were added to all tubes. The tubes were shaken with a vortex mixer for 40 s and centrifuged for 15 min at 2400*g*. The pellet obtained was separated, washed two times with 1 mL of ethanol: ethyl acetate (1:1, volume/volume), and dried at room temperature for 2 min. The pellet was immediately dissolved in 1 mL of denaturizing buffer solution with mixing. Absorbance was measured at 370 nm. Results were expressed as carbonyl content (nmol carbonyl content/mg protein). Trolox (final concentration 1–100 μM) was used as positive control.

2.4.3. Dehydroascorbate (DHA) reductase-like assay

The DHA reductase-like activity of BPIS was assayed as described previously with minor modifications [50,49]. In brief, 10 μL of BPIS at different concentrations (final concentrations of 0.1–25 μM) were incubated (2 min) with 955 μL of 100 mM sodium phosphate buffer, pH 6.9, at 25 °C in the presence or

absence of 10 µL of 100–300 mM GSH (final concentrations of 1–3 mM, diluted in water) in a final volume of 1 mL. The mixture was incubated at 25 °C for 2 min. The DHA reductase assay was initiated by adding 25 µL of 20 mM DHA to a final volume of 1.0 mL. DHA solution was prepared on the day of experiments. For this end, ascorbic acid was added to a solution containing 10 mM sodium phosphate dibasic and 0.5 mM EDTA to achieve a final concentration of 20 mM ascorbic acid. The pH of mixture was adjusted to 5.5 with NaOH. After that, 10 µL of bromine to each 2 mL of ascorbic acid solution pH 5.5 were added and mixed at room temperature for 30 s. Afterward, the solution was bubbled in argon for 10 min. The DHA solution obtained was stored protected from light in ice for up to 4 h. Ascorbic acid regeneration from DHA was recorded at 265 nm. A blank without BPIS was run, and the difference gave the BPIS DHA reductase activity in nmol/min using the molar extinction coefficient of ascorbic acid of 14,700 $cm^{-1}M^{-1}$. Ebselen (final concentration 1–25 µM) was used as positive control.

2.4.4. Glutathione S-transferase (GST)-like assay

The reaction of GSH with CDNB is typically the preferred system used to measure the catalysis imparted by naturally occurring GSTs [14]. An aliquot of 10 µL of BPIS at different concentrations (final concentrations of 0.1–25 µM) was incubated with 20 µL of 50 mM GSH and 950 µL of 100 mM sodium phosphate buffer, pH 6.9 at 25 °C for 3 min. The reaction was initiated by adding 20 µL of 25 mM CDNB to achieve a final volume of 1.0 mL and recorded for 3 min at 340 nm. This assay was also performed without GSH in order to discard possible direct reaction of CDNB with BPIS. CDNB was used as substrate. A blank without BPIS was included and the difference was expressed as Δabs (delta absorbance)/min. Ebselen (final concentration 1–25 µM) was used as positive control.

2.4.5. Scavenging activity of ABTS radical

The determination of the ABTS radical scavenging activity was performed according to the method previously described with some modifications [38]. Initially, the ABTS radical was generated by reacting 7 mM ABTS solution in water with 140 mM potassium persulfate in the dark for 12–16 h. In the day of the assay, the preformed ABTS radical solution was diluted in potassium phosphate buffer in a proportion of 1:88 (1 mL ABTS radical + 87 mL 10 mM potassium phosphate buffer, pH 7.0). Briefly, 1 mL of ABTS radical solution was added to tubes containing 10 µL of BPIS at different concentrations (final concentrations of 0.1–100 µM). The mixture was incubated at 25 °C for 30 min in dark. The decrease in absorbance was measured at 734 nm. Ascorbic acid (1–25 µM) was used as a positive control. Results were expressed as percentage of the control. Ascorbic acid (final concentration 0.1–100 µM) was used as positive control.

2.4.6. Scavenging activity of DPPH radical

Radical-scavenging activity was determined by the reaction of the stable DPPH radical with the compound in accordance with the method previously described [8]. An aliquot of 10 µL of BPIS at different concentrations (final concentrations of 0.1–100 µM) was mixed with 1 mL of methanolic solution containing DPPH radical, resulting in a final concentration of 85 µM DPPH. The mixture was left to stand for 30 min at room temperature in the dark and the absorbance was measured at 517 nm. Results are expressed as percentage of the control. Ascorbic acid (final concentration 0.1–100 µM) was used as positive control.

2.4.7. Scavenging activity of NO and related species (NO_x)

The procedure is based on the principle that SNP in aqueous solution at physiological pH spontaneously generates NO. For the experiment, SNP (10 mM) was mixed with different concentrations of BPIS (final concentrations of 1–100 µM) and incubated at room temperature for 150 min. The same reaction mixture, without the compounds but with an equivalent amount of water, served as the control. After the incubation period, 0.5 mL of Griess reagent (1% sulfanilamide, 2% phosphoric acid and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The NO generated interacts with oxygen to produce nitrite ions that can be estimated using this reagent. Then, NO scavengers could reduce the production of nitrite ions. The absorbance of the chromophore formed was read at 540 nm [28]. The oxime butane-2,3-dionethiosemicarbazone (OXIME; final concentration 25 µM) was used as positive control [37].

2.4.8. δ-ALA-D activity

δ-ALA-D activity was assayed according to the method described by Sassa [41], with some modifications. S_1 (200 µL) was pre-incubated for 10 min at 37 °C in the presence of BPIS at different concentrations (final concentrations of 1–25 µM) or DMSO in the control tube. The enzymatic reaction was initiated by adding the substrate (δ-ALA) to a final concentration of 2.2 mM in a medium containing 45 mM phosphate buffer, pH 6.8 and incubated for 3 h at 37 °C. The incubation was stopped by adding 10% trichloroacetic acid solution (TCA) with 10 mM $HgCl_2$. The reaction product (porphobilinogen) was measured at 555 nm using modified Ehrlich's reagent. The values are expressed as nmol PBG/mg protein/h.

2.4.9. Na^+, K^+-ATPase activity

The reaction mixture for Na^+, K^+-ATPase activity assay contained 3 mM $MgCl_2$, 125 mM NaCl, 20 mM KCl, and 50 mM Tris/HCl, pH 7.4, in a final volume of 500 µL. S_1 (50 µL) was pre-incubated at 37 °C for 10 min in the presence of BPIS (final concentrations of 1–100 µM) or DMSO. The reaction was initiated by the addition of ATP to a final concentration of 3.0 mM and incubated at 37 °C for 30 min. For obtaining the ouabain-sensitive activity, other samples were carried out under the same conditions with the addition of 0.1 mM ouabain. Na^+, K^+-ATPase activity was calculated by the difference between the two assays. Released inorganic phosphate (Pi) was measured by the method of Fiske and Subbarow [13]. The values are expressed as nmol Pi/mg protein/h.

2.4.10. [^{3}H]glutamate uptake by synaptosomes

[^{3}H]glutamate uptake was assayed according to [45]. The synaptosomal preparation was washed twice by suspending in three volumes of 0.3 M sucrose, in 15 mM Tris/acetate buffer (pH 7.4), and centrifuging at 35,000*g* for 15 min. The final pellet was suspended in 0.3 M sucrose, 15 mM Tris/acetate buffer (pH 7.4), and incubated in Tris/HCl buffer (composition in mM Tris/HCl 27, NaCl 133, KCl 2.4, $MgSO_4$ 1.2, KH_2PO_4 1.2, Glucose 12, $CaCl_2$ 1.0) pH 7.4 (adjusted with HCl), in the presence of BPIS (final concentrations of 1.75–5 µM) or DMSO for 10 min at 37 °C. The [^{3}H]glutamate uptake was initiated by adding to the medium [^{3}H]glutamate (final concentration 100 mM) for 1 min at 37 °C. The reaction was stopped by centrifugation (16,000*g*, 1 min, 4 °C), and the pellets were washed three times in Tris/HCl buffer by centrifugation at 16,000*g* for 1 min (at 4 °C). Radioactivity present in pellet was measured in a scintillation counter. Specific [^{3}H]glutamate uptake was calculated as the difference between the uptake obtained in the incubation medium described above, and the uptake obtained with a similar incubation medium in which NaCl was replaced by choline chloride.

2.5. In vivo experiments

Considering that the *in vitro* experiments pointed out the antioxidant properties of BPIS, the compound was used to

investigate its antioxidant effect against damage induced by SNP in the brain of C57BL/6 mice.

2.5.1. Protocol of Exposure

Mice were divided into 4 groups of 7–9 animals each. Animals belonging to groups I and III received oral application of canola oil (10 mL/kg of body weight). Mice of groups II and IV received oral administration of BPIS (10 mg/kg). Thirty minutes after the treatment, mice of groups III and IV received SNP (0.335 μmol per site/2 μL) intracerebroventricular (i.c.v.). Groups I and II received saline solution (i.c.v.). The dosage of SNP was based on our previous study [36] and the BPIS dose was selected based on our previous study that demonstrated the antinociceptive action of BPIS through a series of acute models [7]. I.c.v. injections were given as described previously [22], with the bregma fissure as a reference point.

2.5.2. Tissue preparation

One hour after SNP or saline administration all mice were kiled and the brains of animals were removed and homogenized as described in Section 2.3. The low-speed supernatants (S_1) were separated and used for *in vivo* assays. For protein carbonyl content, it was used the homogenate without centrifugation. In addition for NO_x levels, the brains were homogenized with $ZnSO_4$ (200 mM) and acetonitrile (96%), centrifuged at 16,000g at 4 °C for 30 min, and the supernatant was collected.

2.5.3. Lipid peroxidation

Lipid peroxidation was carried out with an aliquot of S_1 (200 μL) as described in Section 2.4.1, excepting for the absence of the preincubation step.

2.5.4. Reactive species (RS) determination

RS levels were determined by a spectrofluorimetric method, using DCHF-DA assay [25]. S_1 (10 μL) was incubated with 10 μL of DCHF-DA (1 mM). The oxidation of DCHF-DA to fluorescent dichlorofluorescein (DCF) is measured for the detection of intracellular RS. The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) 30 min after the addition of DCHF-DA to the medium. RS levels were expressed as arbitrary units (AU) of fluorescence/g tissue.

2.5.5. Non-protein thiol (NPSH) content

NPSH levels were determined by the method previously described [10]. To determine NPSH, S_1 was mixed (1:1) with 10% trichloroacetic acid. After the centrifugation, the protein pellet was discarded and free –SH groups were determined in the clear supernatant. An aliquot of supernatant was added in 1 M potassium phosphate buffer pH 7.4 and 10 mM DTNB. The color reaction was measured at 412 nm. NPSH levels were expressed as optic density (DO)/g tissue.

2.5.6. Protein carbonyl determination

Protein carbonyl content was carried out with an aliquot of 940 μL of a homogenate diluted with Tris–HCl buffer, pH 7.4 in a proportion of 1:8, as described above Section 2.4.2, excepting for the absence of the preincubation step.

2.5.7. NO_x levels

The brains were dissected on an inverted ice-cold Petri dish and homogenized with $ZnSO_4$ (200 mM) and acetonitrile (96%), centrifuged at 16,000g at 4 °C for 30 min, and the supernatant was collected for assay of the nitrite plus nitrate content [28]. NO_x content was estimated in a medium containing 900 μL of the previously described Griess Reagent. After incubating at 37 °C for 60 min, nitrite levels were determined spectrophotometrically at 540 nm, based on NO_x/g tissue.

2.5.8. Catalase (CAT) activity

Enzymatic reaction was initiated by adding an aliquot of 20 μL of the S_1 and the substrate (H_2O_2) to a concentration of 0.3 mM in a medium containing 50 mM phosphate buffer, pH 7.5. The enzymatic activity was measured at 240 nm and expressed as Unit (U)/mg protein (1 U decomposes 1 μmol of H_2O_2 per minute at pH 7 at 25 °C) [1].

2.5.9. Superoxide dismutase (SOD) activity

S_1 was diluted 1:10 (v/v) for determination of SOD activity in the test day. Aliquots of supernatant were added in a Na_2CO_3 buffer 50 mM pH 10.3. Enzymatic reaction was started by adding of epinephrine. The color reaction was spectrophotometrically measured at 480 nm. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50% at 26 °C [29]. The enzymatic activity was expressed as U/mg protein.

2.5.10. Glutathione-S-trasferase (GST) activity

GST activity was assayed spectrophotometrically at 340 nm by the method of Habig et al. [14]. The reaction mixture contained an aliquot of S_1, 0.1 M potassium phosphate buffer pH 7.4, 100 mM GSH and 100 mM CDNB, which was used as substrate. The enzymatic activity was expressed as nmol CDNB conjugate/min/mg ptn.

2.5.11. Glutathione reductase (GR) activity

GR activity in S_1 was determined as described by Calberg and Mannervik [6]. In this assay, GSSG is reduced by GR at the expense of NADPH consumption, which was followed at 340 nm. GR activity is proportional to NADPH decay. The enzymatic activity was expressed as nmol NADPH/min/mg protein.

2.5.12. Glutathione peroxidase (GPx) activity

GPx activity in S_1 was assayed spectrophotometrically by the method of Wendel [51], through the GSH/NADPH/glutathione reductase system, by the dismutation of H_2O_2 at 340 nm. S_1 was added in GSH/NADPH/glutathione reductase system and the enzymatic reaction was initiated by adding H_2O_2. In this assay, the enzyme activity was indirectly measured by means of NADPH decay. H_2O_2 is reduced and generates GSSG from GSH. GSSG is regenerated back to GSH by glutathione reductase present in the assay media at the expenses of NADPH. The enzymatic activity was expressed as nmol NADPH/min/mg protein.

2.6. Protein quantification

Protein concentration was measured by the method of Bradford [3], using bovine serum albumin (1 mg/mL) as the standard.

2.7. Statistical analysis

The results are presented as mean ± SEM. For *in vitro* experiments, the data were performed using one-way analysis of variance followed by the Newman–Keul's multiple range test when appropriate. The IC_{50} values were calculated considering responses between 20 and 80% and reported as geometric means accompanied by their 95% confidence limits, using the program GraphPad InSTAT. Maximal inhibition (I_{MAX}) values were calculated at the most effective dose used. For *in vivo* experiments, data were analyzed using two-way analysis of variance (ANOVA) followed by the Newman–Keul's multiple range test when appropriate. p values less than 0.05 ($p < 0.05$) were considered as indicative of significance.

3. Results

3.1. In vitro experiments

3.1.1. Effect of BPIS on Fe (II)/EDTA-induced TBARS in rat brain homogenate

BPIS significantly reduced the lipid peroxidation induced in rat brain homogenate at concentrations equal or greater than 1 μM [$F_{(7,16)} = 36.03$; $p < 0.001$] (Fig. 2A). The IC_{50} value was 1.35 (1.29–1.42) μM and I_{MAX} 89 ± 1%. The positive control ascorbic acid was only effective in the concentration of 1000 μM with I_{MAX} 38 ± 6% (data not shown).

3.1.2. Effect of BPIS on protein carbonyl production induced by SNP in rat brain homogenate

As seen in Fig. 2B, BPIS decreased the protein carbonyl levels induced by SNP in rat brain homogenate at concentrations equal or greater than 0.5 μM [$F_{(6,14)} = 39.58$; $p < 0.001$]. The IC_{50} value was 0.74 (0.70–0.77) μM and I_{MAX} 65 ± 5%. The positive control trolox was effective at concentrations equal or greater than 50 μM with I_{MAX} 32 ± 2% (data not shown).

3.1.3. DHA-Reductase-like activity of BPIS

BPIS, at concentration equal or greater than 5 μM demonstrated DHA-Reductase-like activity, being effective to reduce DHA to

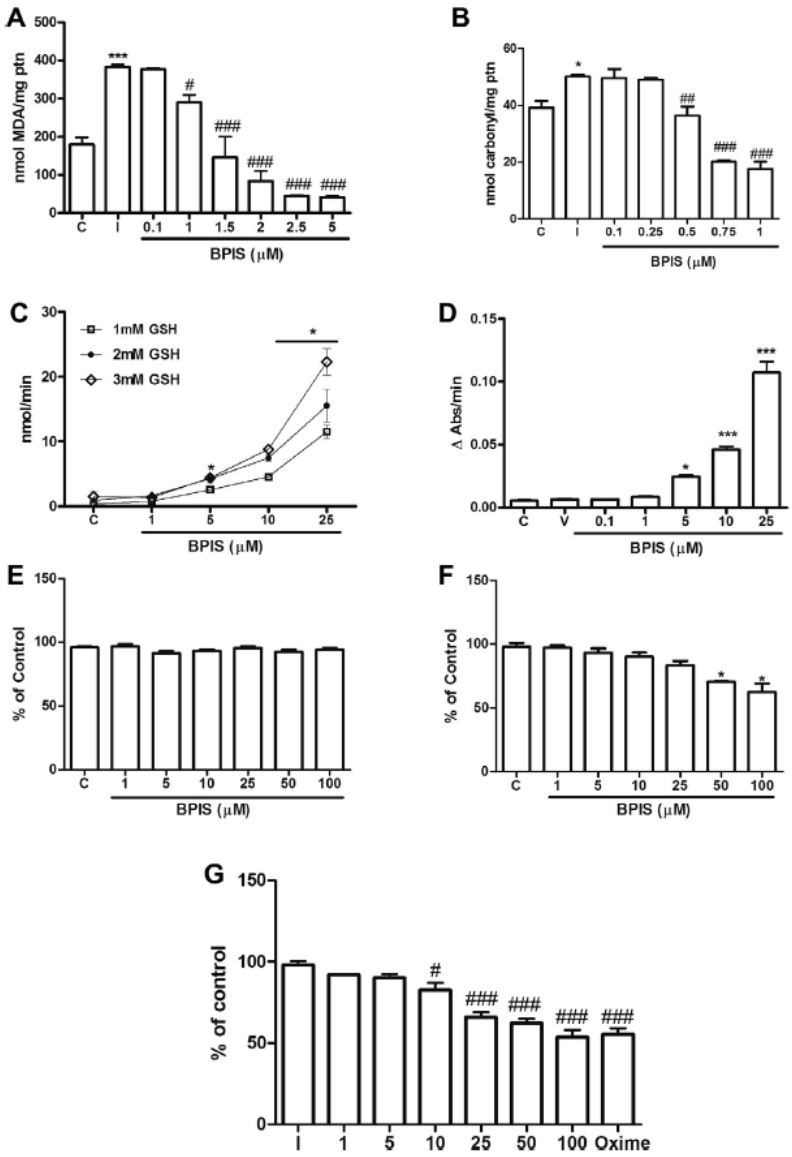

Fig. 2. Effect of BPIS on (A) lipid peroxidation induced by Fe(II)/EDTA and (B) protein carbonyl production induced by SNP in rat brain homogenates *in vitro*; (C) DHA-Reductase-like and (D) GST-like activities of BPIS; (E) ABTS, DPPH and NO_x-scavenging activities of BPIS. Data are reported as mean ± SEM for 3–4 experiments performed in duplicate, in different days, using different animals. (*) denotes $p < 0.05$ as compared to the control; (***) denotes $p < 0.001$ as compared to the control; (#) denotes $p < 0.05$ as compared to the induced; (##) denotes $p < 0.01$ as compared to the induced; (###) denotes $p < 0.001$ as compared to the induced (one way ANOVA/Newman–Keuls).

ascorbic acid. The rate of reduction was proportional to the concentration of BPIS, but similar to the different concentrations of GSH (Fig. 2C): in the presence of 1 mM of GSH [$F_{(6,14)} = 86.45$; $p < 0.001$]; 2 mM of GSH [$F_{(6,14)} = 29.24$; $p < 0.001$] and 3 mM of GSH [$F_{(6,14)} = 73.18$; $p < 0.001$]. The assay was also performed without GSH, and no ascorbic acid formation was observed, discarding any direct reaction of BPIS directly to DHA (data not shown).

The positive control ebselen did not show DHA-Reductase-like activity in the presence of 1 mM of GSH. In the presence of 2 mM of GSH, ebselen showed DHA-Reductase-like activity at concentration equal or greater than 10 μM. At last, in the presence of 3 mM of GSH, ebselen showed DHA-Reductase-like activity at concentration equal or greater than 5 μM (data not shown).

3.1.4. GST-like activity of BPIS

The data show that BPIS had GST-like activity in concentrations equal or greater than 5 μM [$F_{(5,12)} = 103.0$; $p < 0.01$] (Fig. 2D). The assay was also performed without GSH, and no product formation was observed, discarding any direct reaction of BPIS directly to CDNB (data not shown). The positive control ebselen only showed a significant GST-like activity at the concentration of 25 μM (data not shown).

3.1.5. ABTS and DPPH radical-scavenging activity

As seen in Fig. 2E, BPIS did not show ABTS radical scavenging activity at assayed concentrations [$F_{(6,14)} = 1.952$; $p = 0.1418$]. By contrast, BPIS showed DPPH radical scavenging activity at concentrations equal or greater than 50 μM [$F_{(6,14)} = 14.79$; $p < 0.001$] (Fig. 2F). For the DPPH assay, the IC_{50} value was not calculated considering that the I_{MAX} was 36 ± 7%.

The positive control ascorbic acid showed both ABTS and DPPH radical-scavenging activity at concentrations equal or greater than 5 μM (data not shown). For the ABTS assay the IC_{50} value was 8.63 μM and I_{MAX} was 96 ± 1% and for DPPH assay the IC_{50} value was 9.01 μM and I_{MAX} was 93 ± 2%.

3.1.6. NO_x-scavenging activity

BPIS diminuished the NO and related species formation induced by SNP at concentrations equal or greater than 10 μM [$F_{(7,16)} = 32.63$; $p < 0.001$] (Fig. 2G). The IC_{50} value was not calculated considering that the I_{MAX} was 45 ± 4%. OXIME was evaluated only in the concentration of 25 μM, it presented inhibition of 43 ± 4%.

3.1.7. Effect of BPIS on δ-ALA-D Activity in rat brain homogenate

BPIS significantly reduced the δ-ALA-D activity from rat brain homogenate at concentrations equal or greater than 10 μM [$F_{(4,10)} = 15.82$; $p < 0.001$] (Fig. 3A). The IC_{50} value was 16.41 μM (15.25–17.65) and I_{MAX} was 74 ± 14%.

3.1.8. Effect of BPIS on Na^+, K^+-ATPase activity in rat brain homogenate

As seen in Fig. 3B, BPIS showed a dual effect on the Na^+, K^+-ATPase activity in rat brain homogenates, significantly acting as an enzyme inductor at low concentrations (i.e. 1–10 μM) and as an inhibitor at high concentrations (i.e. equal or greater than 50 μM) [$F_{(6,14)} = 37.96$; $p < 0.001$]. The IC_{50} value was 26.44 (25.55–27.36) μM and I_{MAX} value was 86 ± 10%.

3.1.9. Effect of BPIS on [^{3}H]glutamate uptake by synaptosomes

BPIS significantly inhibited [^{3}H]glutamate uptake by synaptosomes at concentrations equal or greater than 3.75 μM [$F_{(4,10)} = 10.52$; $p < 0.01$] (Fig. 3C). The IC_{50} value was 3.294 μM and I_{MAX} was 74 ± 10%.

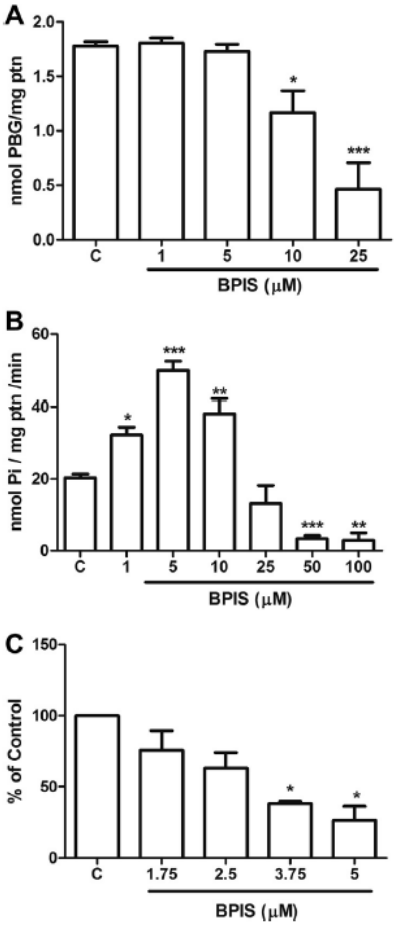

Fig. 3. Effect of BPIS on (A) δ-ALA-D, (B) Na^+, K^+-ATPase activities in rat brain homogenates and (C) [^{3}H]glutamate uptake by synaptosomes *in vitro*. Data are reported as mean ± SEM for 3–5 experiments performed in duplicate, in different days, using different animals. (*) denotes $p < 0.05$ as compared to the control; (**) denotes $p < 0.01$ as compared to the control; (***) denotes $p < 0.001$ as compared to the control (one way ANOVA/Newman–Keuls).

3.2. In vivo experiments

3.2.1. Lipid peroxidation

Two-way ANOVA of TBARS determination yielded a significant SNP × BPIS interaction [$F_{(1,26)} = 5.956$; $p < 0.05$]. Post-hoc comparisons demonstrated that SNP significantly induced lipid peroxidation in brains of mice and BPIS protected against the increase in these levels (Fig. 4A).

3.2.2. RS levels

According to two-way ANOVA, RS levels were not modified in rats in none of the experimental groups [$F_{(1,26)} = 0.011$; $p = 0.973$] (Fig. 4B).

3.2.3. NPSH levels

Regarding the NPSH levels, the two-way ANOVA showed a significant interaction between SNP and BPIS [$F_{(1,26)} = 4.291$; $p < 0.05$]. The BPIS pretreatment was effective in preventing the decrease in NPSH levels caused by SNP i.c.v. injection in brains of mice (Fig. 4C).

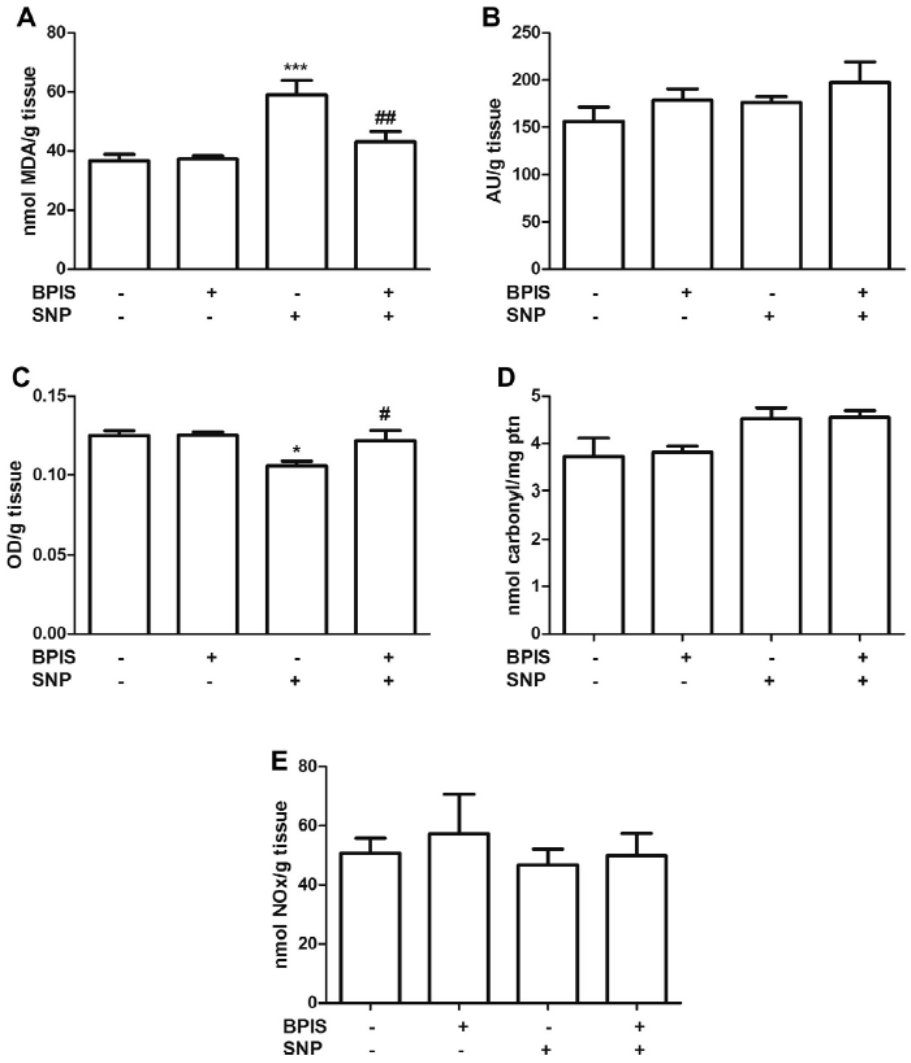

Fig. 4. Effect of BPIS (10 mg/kg) on (A) lipid peroxidation, (B) RS, (C) NPSH, (D) protein carbonyl and (E) NO_x levels in brains of mice treated with SNP. Data are reported as mean ± SEM of 7–9 animals per group. (*) denotes $p < 0.05$ as compared to the control group; (***) denotes $p < 0.001$ as compared to the control group; (#) denotes $p < 0.05$ as compared to the SNP group; (##) denotes $p < 0.01$ as compared to the SNP group (two-way ANOVA/Newman–Keuls).

3.2.4. Protein carbonyl content

In relation to carbonyl content, the two-way analysis revealed that there a main effect of SNP i.c.v. injection [$F_{(1,26)} = 10.579$; $p < 0.05$]; however, there was no interaction between SNP and BPIS treatments [$F_{(1,26)} = 0.018$; $p = 0.894$] (Fig. 4D).

3.2.5. NO_x levels

As seen in Fig. 4E, two-way ANOVA indicated that the administration of SNP and/or BPIS did not change NO_x levels in the brain of mice [$F_{(1,26)} = 0.049$; $p = 0.827$].

3.2.6. Antioxidant enzyme activities

Two-way analysis demonstrated that there was no significant interaction between SNP and BPIS treatments for CAT [$F_{(1,26)} = 0.068$; $p = 0.796$] (Fig. 5A), SOD [$F_{(1,26)} = 0.150$; $p = 0.702$] (Fig. 5B), GR [$F_{(1,26)} = 2.930$; $p = 0.099$] (Fig. 5C) and GST activities [$F_{(1,26)} = 2.464$; $p = 0.129$] (Fig. 5D).

Regarding GPx activity, two-way ANOVA revealed a signifcant main effect of BPIS treatment [$F_{(1,26)} = 5.106$; $p < 0.05$] (Fig. 5E). Although there was no significant interaction between SNP and BPIS treatments [$F_{(1,26)} = 3.53$; $p = 0.071$], GPx data from animals that received both BPIS and SNP was different from all the other groups.

4. Discussion

The results of the present study demonstrate that the compound BPIS had an *in vitro* and *in vivo* antioxidant activity. *In vitro*, BPIS protected against lipid peroxidation induced by Fe (II)/EDTA and protein carbonyl formation induced by SNP in rat brain homogenate. The mechanism of action by which BPIS shows antioxidant

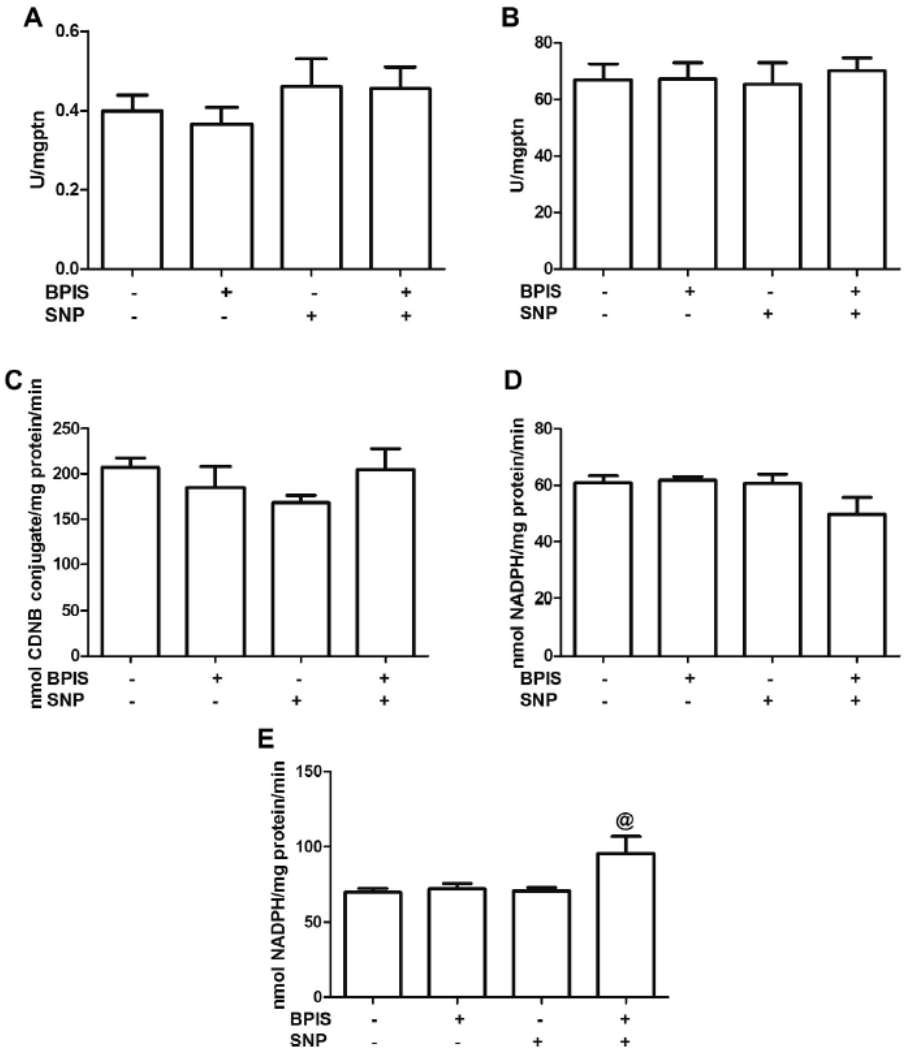

Fig. 5. Effect of BPIS (10 mg/kg) on (A) CAT, (B) SOD, (C) GST, (D) GR and (E) GPx activity in brains of mice treated with SNP. Data are reported as mean ± SD of seven animals per group. Data are reported as mean ± SEM of 7–9 animals per group. (@) denotes $p < 0.05$ as compared to all the other groups (two-way ANOVA/Newman–Keuls).

activity is related to its GST-like and DHA-Reductase-like activities as well as DPPH and NO-scavenging activity. It was also demonstrated, that BPIS can inhibit enzymes such as δ-ALA-d and Na^+, K^+-ATPase and the glutamate uptake, which could represent a toxicological potential, however, these effects were demonstrated to occur at higher concentrations than those of it showed antioxidant potential. In *in vivo* experiments, BPIS protected against alterations in parameters of oxidative stress induced by i.c.v. SNP administration, strengthening the BPIS potential as an antioxidant compound.

It is known that basal levels of ROS and RNS are physiologically produced by several mechanisms, including partial reduction of O_2 via mitochondrial electron transport chain, the oxidative deamination of biogenic amines and as part of the immune response by polymorphonuclear cells [4,11]. ROS and RNS are generally detoxified by enzymatic and non-enzymatic antioxidant defenses, maintaining the natural redox status of the cells [16]. However, when there is an imbalance between the production of RS and the natural antioxidant defenses, this situation is considered oxidative stress. Diverse molecules can be oxidized due to an augment in RS levels, among these macromolecules, we can mention protein, lipids and nucleic acids, generating cellular damage [34].

Lipid peroxidation and protein carbonylation can be observed in situations like aging, cancer and neurodegeneration [17,4,46]. The brain is extremely susceptible to oxidative stress, especially because many areas of this organ are rich in non-haem iron, which is catalytically involved in the generation of free radicals; as well as the brain contains high degree of polyunsaturated fatty acids, substrates particularly vulnerable to oxidation [15]. For this reason, we investigated the *in vitro* antioxidant effect of BPIS in brain homogenates, and the results demonstrated that BPIS acts as an antioxidant at very low concentrations: The results demonstrated IC_{50} values of 1.35 μM in the lipid peroxidation induced by Fe(II)/EDTA and 0.74 μM in the protein carbonyl production induced by SNP. Studies have demonstrated organoselenium compounds,

among them diaryl diselenides, as promising antioxidant agents against models of oxidative stress [27,36,2].

However, BPIS showed lower IC_{50} values when compared to other diselenides: for example, Prigol et al. [36] demonstrated that among many disubstituted diaryl diselenides tested, *p*-chlorodiphenyl diselenide $(p\text{-ClPhSe})_2$ showed the best antioxidant profile (IC_{50} 1.90 μM for SNP-induced lipid peroxidation, 2.7 μM for malonate-induced lipid peroxidation and 85 μM for SNP-induced protein carbonyl formation). Dithienyl diselenide $(ThSe)_2$, another parental compound, presented IC_{50} values of 15.33 μM for FE(II)/EDTA-induced lipid peroxidation and 11.89 μM for SNP-induced protein carbonyl formation.

Although the precise mechanism by which BPIS elicits antioxidant activity is still not completely understood, a large part of its action is the mimetic activity of the physiological enzymes DHA-Reductase and GST. DHA-Reductase is the enzyme responsible for the recycling of dehydroascorbate into ascorbic acid, one of the most important physiological antioxidants and GST is a detoxifying enzyme for xenobiotics, lipid peroxidation end products and other oxidative stress-related molecules [50,43]. BPIS acts similar to these enzymes, using GSH as a reducing agent in order to prevent oxidative stress, then contributing to ascorbic acid recycling and thiol-dependent xenobiotic detoxification.

In addition, BPIS showed scavenging activity against free radicals, represented by the ability to scavenge DPPH, as well as NO and related compounds. ABTS and DPPH radical-scavenging activity assays are widely used as antioxidant activity screening, differences in the scavenging activity can be due to DPPH reactions involve H-atom transfer and reactions with ABTS radicals involve electron-transfer processes [30]. BPIS also demonstrated NO_x scavenging activity, it is known that NO can undergo reactions with O_2, superoxide ion and reducing agents to produce RNS, such as nitroxyl (HNO), the oxides NO_2/N_2O_4, and N_2O_3, peroxynitrite, and S-nitrosothiols (RSNO), molecules that contribute to nitrosative and oxidative stress [19]. We have to mention that the concentrations in which BPIS showed scavenger activities were higher than those in which BPIS elicited antioxidant activity, but these could be contributing to the sum of antioxidant properties of this compound.

The inhibition of sulfhydryl enzymes, such as δ-ALA-d and Na^+, K^+-ATPase activities, and the blockade of glutamate uptake can represent important points to be investigate related to the toxicity of organoselenium compounds. The interaction with these enzymes or transporter is associated with the fact that most of organoselenium compounds are highly prone to attack thiol groups present in these proteins [32]. δ-ALA-D is an important enzyme related to haem biosynthesis, Na^+, K^+-ATPase is responsible for the active transport of sodium and potassium ions in the central nervous system and the glutamate uptake, carried out by excitatory amino-acid transporters, regulates concentrations of glutamate in the extracellular space [41,48,47]. The inhibition of these pathways generally leads to excitotoxicity, a situation reported in toxicity caused by high concentrations of organoselenium compounds [31]. However, we have to highlight that these parameters were mainly affected at higher concentrations than BPIS showed antioxidant effect. Other interesting fact is that in low concentrations, BPIS even stimulated the Na^+, K^+-ATPase activity; considering that oxidative regulation of this enzyme has important implications, reducing agents could increase the Na^+, K^+-ATPase activity or reverse the physiological inhibition caused by normal oxidative status [12].

Based on the *in vitro* results, we expanded the study in order to investigate the effect of an oral administration of BPIS on the model of oxidative damage induced by i.c.v. injection of SNP in mice. It was demonstrated that BPIS prevented against SNP-mediated alterations in oxidative stress parameters in brains of mice. BPIS avoided the increase in lipid peroxidation levels and the decrease in NPSH levels induced by the injection of SNP. SNP treatment did not affect all of the analyzed parameters, but the oxidation of protein, lipids and endogenous thiols is something to be highlighted, whereas that these are important parameters of oxidative stress [35].

Regarding that there is no significant alteration in the levels of RS or NO_x in the animals treated with SNP, it could be reacting more directly to lipids, thiols and proteins. We could not discard that other species related to SNP and NO metabolism could be elevated, whereas that the griess reagent mainly reacts with NO_2 and NO_3. The greater prevalence and reactivity of thiols over other biological nucleophiles could explain the propensity for S-nitrosothiol formation, what could explain the decrease in NPSH in the groups that received only SNP. Thiols in the presence of electron acceptors (redox-activated thiols) react with the nitrosonium moiety of NO to form S-nitrosothiol which may represent a storage pool for NO [19]. As already mentioned, SNP release cyanide and iron from its structure, what induce reduction of enzyme mitochondrial activity as well as production of ROS and RNS, triggering to oxidative stress and damage, represented by the oxidation of macromolecules [21,24]. We cannot affirm, regarding that we do not know the concentrations of BPIS in the brain tissue, when administered orally, but we can suppose that it could be acting detoxifying the SNP-induced damage by mechanisms similar to observed *in vitro*, such as GST- and DHA-Reductase-like, as wells as free radicals-scavenging activities, especially to NO-related molecules.

SNP did not affect the activity of the evaluated antioxidant enzymes (CAT, SOD, GR, GST and GPx), an important point to be investigated, but that seems not to be related to the toxic effects of SNP. Despite this, when the animals received both BPIS and SNP, the GPx activity was increased. It is known that some organoselenium compounds could stimulate the expression of GPx, and some organoselenium (e.g. selenocystein) can even be incorporated in selenoproteins as GPx [32,18]. However, the time following exposure to SNP and/or BPIS and tissue extraction is insufficient for induction of the enzyme expression, and it seems to merely reflects enzyme activation, this could also explain why some enzyme activities were not affected by the treatments. GPx is an antioxidant selenoenzyme that reduces variable hydroperoxides at the expense of glutathione and/or other reducing equivalents [18]. One interesting point is that both situations were necessary to increase GPx activity, just BPIS administration does not increase its activity, but also in association with a situation of oxidative damage.

Some of the limitations of this study include the dose of SNP which does not generate much harm, even if the results related to oxidative damage are similar to those observed in other studies [36,44]. In addition, the *in vitro* data cannot be extrapolated to the *in vivo* because the blood or brain concentrations of BPIS were not determined after oral administration of this compound to mice and in view of the fact that these experiments were carried out with species differentes. Despite these limitations, the present study provides valuable information for the *in vitro* and *in vivo* antioxidant potential of BPIS.

5. Conclusions

In conclusion, the results demonstrated the *in vitro* antioxidant action of BPIS on rat brain homogenates, at low concentrations, and this could be explained for its ability to mimic physiological antioxidant enzymes, as well as scavenge free radicals. BPIS administered to mice protected against alterations in parameters of oxidative stress caused by SNP in mouse brain, strengthening the potential antioxidant effect of this compound. Although the results

showed here indicate that BPIS might be a good candidate for future drug development in the prevention or treatment of diseases related to oxidative stress, more results would be required for better understanding the complete mechanism by which this compound acts and its physiological effects.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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3.3 Manuscrito 1

Eficácia do Disseleneto de Bis(fenilimidazoselenazolila) em um Modelo de Nocicepção Inflamatória em Camundongos: Perspectivas do Mecanismo de Ação Espinhal

EFFECTIVENESS OF BIS(PHENYLIMIDAZOSELENAZOLYL) DISELENIDE ON A MOUSE MODEL OF INFLAMMATORY NOCICEPTION: INSIGHTS ON THE MECHANISM OF SPINAL ACTION

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Effectiveness of bis(phenylimidazoselenazolyl) diselenide on a mouse model of inflammatory nociception: Insights on the mechanism of spinal action

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Abstract

The injection of complete Freund's adjuvant (CFA) in the hindpaw of rodents induces tissue inflammation and nociceptive hypersensitivity. In addition, it has been reported that organoselenium compounds have antinociceptive properties in animal models. The purpose of this study was to investigate the potential protective effect of bis(phenylimidazoselenazolyl) diselenide (BPIS) in the inflammatory nociception model in mice and its possible mechanism of action. C57BL/6 mice received CFA i.pl. in right hindpaw and the inflammatory response was verified 24h after injection as well as the protective effect of BPIS. The CFA-induced mechanical allodynia was reversed by BPIS treatment (1 mg/kg, p.o.) observed through von Frey hair Test. Additionally, L-arginine (600 mg/kg; i.p.; nitric oxide precursor) before BPIS treatment, prevented the antinociceptive effect. In tissue analysis, whereas alterations observed in paw of animals injected with CFA were not reversed by BPIS administration, BPIS reversed the increase in spinal NOx content induced by CFA. In the spinal cord, it was also found that CFA induced an increase in malondialdehyde content and a decrease in glutamate uptake, and these alterations were reversed by BPIS. Moreover, BPIS treatment induced an increase in non-protein thiol levels in animals that received CFA injection. The obtained data reinforce the relevance of BPIS, a novel organoselenium compound, as a potential antinociceptive agent as well as highlights the importance of the nitric oxide pathway in the spinal cord and its antioxidant potential for its mechanism of action.

Keywords: selenium; nociception; nitric oxide; spinal cord

1 Introduction

Persistent pain may lead to emotional negative reactions, turning into a debilitating and suffering causer (CHAPMAN e GAVRIN, 1999). The nociceptive response is controlled by a wide range of mediators that interact with neurotransmitter and modulator proteins (JULIUS e BASBAUM, 2001). Many inflammatory mediators have been recognized for sensitizing nociceptive neurons (KIDD e URBAN, 2001). It is also known that pain related to inflamed tissues may be related to hypersensitivity (LINLEY et al., 2010).

Throughout the immune response, leucocytes produce high amount of oxidant reactive species, which if left uncontrolled can worse inflammation and tissue damage. Additionally, there is evidence that the antioxidant system also exerts important effect in regulation of pain and inflammation (VALKO et al., 2007). Studies have shown that selenium supplementation is effective in modulating inflammation and immunity, especially because of the involvement of selenoproteins in these processes. In addition, selenium insufficiency is related to increased oxidative stress, together with impaired immune cells activity (HUANG et al., 2012).

In this context, special attention can be given to organoselenium compounds, which have been reported to have antioxidant, antinociceptive and anti-inflammatory properties (NOGUEIRA e ROCHA, 2011). Moreover, the organoselenium bis(phenylimidazoselenazolyl) diselenide (BPIS; Figure 1) has exhibited antioxidant *in vitro* and *in vivo* properties, as well as antinociceptive effect in models of thermal and chemical nociception in mice without apparent signs of toxicity (CHAGAS et al., 2013; CHAGAS et al., 2015).

Based on already mentioned properties of BPIS, the objectives of the present study were to investigate the potential protective effect of BPIS on CFA-induced inflammatory nociception and explore its possible action mechanism.

2 Material and Methods

2.1 Chemicals

BPIS was prepared and characterized according to Roehrs et al. (2012). Analysis of the ^{1}H NMR and ^{13}C NMR spectra shed analytical and spectroscopic data in full agreement with its assigned structure. Adenosine 5′-triphosphate (ATP) disodium salt hydrate, 1-chloro-2,4-dinitrobenzene (CNDB), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), CFA, [^{3}H]-L-glutamic acid, glutathione (GSH), β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH), sodium dodecyl sulfate (SDS), thiobarbituric acid (TBA), trichloroacetic acid (TCA), N, N, N', N'-tetramethylbenzidine and vanadium (III) chloride were purchased from the Sigma Chemical Co. (São Paulo, Brazil). All other chemicals were of analytical grade and obtained from standard commercial suppliers. BPIS was dissolved in canola oil for the administration in the animals.

2.2 Animals

Male adult C57BL/6 mice (25-35g) from our own breeding colony were used for the experiments. Animals were kept on a separate animal room, in a 12 h light/dark cycle, at a room temperature of 22 ± 2° C, with free access to food (Guabi, RS, Brazil) and water. The animals were used according to the guidelines of the

Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Brazil (#066/2014).

2.3 CFA-induced Inflammatory Pain Model

It was investigated the effect caused by BPIS on inflammatory pain model, through immunologic reaction induced by intraplantar (i.pl.) injection of CFA. Briefly, the mice received 20 µl of CFA i.pl. (1 mg/ml of heat killed Mycobacterium tuberculosis in 85% paraffin oil and 15% mannide monooleate) into the right hindpaw. The inflammatory response was verified 24 h after CFA injection in the plantar surface of the right hindpaw in mice. After this basal evaluation, we investigated the antinociceptive effect of BPIS as described below. The animal groups were randomized without observer knowledge of treatments during measurement and analysis.

2.3.1 Behavioral Assessment

2.3.1.1 Mechanical Allodynia

The mechanical allodynia was performed as described previously (BORTALANZA et al., 2002). In this experiment, we evaluated the time–course of the antinociceptive effect of BPIS at the doses of 0.1 and 1 mg/kg via oral administration (p.o.). The baseline response was performed 24 h after CFA injection in the plantar surface of the right hindpaw in mice. The frequency of withdrawal was also determined in the animals, which have not received CFA injection, in order to obtain data purely derived from the treatments in CFA hypersensitivity. Mechanical alodynia in mice with CFA was measured by using a calibrated nylon von Frey hair (VFH) filament of 0.6 g (mean of 10 applications for each mice). Therefore, the mice that presented mechanical allodynia received vehicle (canola oil, p.o., 10 ml/kg) or BPIS and the

withdrawal response frequency in VFH was recorded after (0.5, 1, 2, 4, 6 and 8 h) BPIS treatment (N: 6/group). Dexamethasone (DEX, 2 mg/kg; p.o. in saline; N: 6/group) was used as positive control (DE LIMA et al., 2011).

2.3.1.2 Involvement of L-arginine-NO Pathway in the Antinociceptive Effect of BPIS

In order to address the role played by the L-arginine-NO pathway in the antinociceptive effect caused by BPIS (1 mg/kg; p.o.) on the VFH, distinct groups of animals were pre-treated with L-arginine (600 mg/kg, intraperitoneally [i.p.]; a dose that produces no effect on the VFH) or saline (10 ml/kg, i.p.) 24h after CFA injection (YONEHARA et al., 1997). Twenty-minutes after L-arginine administration, the VFH was performed as previously mentioned. For this protocol, the animals were divided in four groups with six animals per group: I. CFA + saline + canola oil; II. CFA + L-arginine + canola oil; III. CFA + saline + BPIS; IV. CFA + L-arginine + BPIS. Similarly, the withdrawal response frequency in VFH was recorded after (0.5, 1, 2, 4, 6 and 8 h) BPIS treatment (1 mg/kg; p.o.).

2.3.3 Tissue Analyses

Only the highest dose of BPIS (1 mg/kg, p.o.) was selected for the tissue analyses, however, all the animals were submitted to the same treatment schedule as described in the section 2.3.1.1, in order to simulate the same alterations observed in the animals submitted to the behavioral tests. The mice received the CFA injection 24 h before BPIS administration and were killed 8h after treatment by decapitation.

The samples of the spinal cord tissue as well as the injected paws (cut below the knee joint) were rapidly dissected, weighed and were frozen at -80 ^{o}C or place on ice for use on the same day. The samples were used to determine myeloperoxidase (MPO) activity, nitrate/nitrite (NOx) and 3-nitrotyrosine (3-NT) content. Samples of spinal cord tissue were also used to determine Na^{+}, K^{+} ATPase activity, [H^{3}]glutamate uptake, oxidative stress parameters.

2.3.3.1 Myeloperoxidase Assay

The MPO activity was assayed according to the method of Suzuki *et al.* (1983) with some modifications. The spinal cord and the right hindpaw tissue were homogenized in potassium phosphate buffer (20 mmol/l, pH 7.4; 1:5, w/v) containing ethylenediaminetetraacetic acid (0.1 mmol/l). After the homogenization, the samples were centrifuged at 2000×g at 4 ^{o}C for 10 min to yield a low-speed supernatant fraction (S_1). Then, the S_1 fraction was centrifuged again at 20000×g at 4 ^{o}C for 15 min to yield a final pellet (P_2) that was resuspended in medium containing potassium phosphate buffer (50 mmol/l, pH 6.0) and hexadecyltrimethyl ammonium bromide (0.5%). The samples were finally frozen and thawed three times for the posterior enzymatic assay.

For the MPO activity measurement, an aliquot of resuspended P_2 (100 µl) was added to a cuvette containing the medium of resuspension and N, N, N', N'-tetramethylbenzidine (1.5 mmol/l). The kinetic analysis of MPO was started after H_2O_2 (0.01%) addition, and the color reaction was measured at 655 nm at 37 ^{o}C. Results are expressed as absorbance (Abs)/mg protein/min (N: 6/group).

2.3.3.2 Determination of NOx content

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The right hindpaw and the spinal cord tissues were homogenized with $ZnSO_4$ (200 mM; 500 µl) and acetonitrile (96%; 500 µl), centrifuged at 16 000×g at 4 °C for 30 min, and the supernatant was collected for assay of the nitrite plus nitrate content (MIRANDA et al., 2001). NOx content was estimated in a medium containing 300 µl of 2% VCl_3 (in 5% HCl), 200 µl of 0.1% N-(1-naphthyl) ethylene-diaminedihydrochloride, and 200 µl of 2% sulfanilamide (in 5% HCl). After incubating at 37 °C for 60 min, nitrite levels were determined spectrophotometrically at 540 nm, based on NOx/g of tissue (N: 6/group).

2.3.3.3 Determination of 3-NT content

Determination of 3-NT and tyrosine (Tyr) was performed by high-performance liquid chromatography coupled to ultraviolet (HPLC-UV) detection method (ERDAL et al., 2008). For the assay, the tissues (paw and spinal cord) were immediately homogenized in cold 50 mM Tris–HCl, pH 7.4 (1/5, w/v). The homogenates were centrifuged at 2400×g for 10 min to yield a low-speed supernatant (S_1). Briefly, S_1 aliquots of each sample were hydrolyzed in HCl (12 N; 1:1 v/v) at 60 °C for 24 h. Digested samples were filtered through a membrane (0.45-µm-pore size) Millipore® before injection on to the HPLC instrument. Samples were analyzed on a Shimadzu® HPLC apparatus. The analytical column was a 5-µm-pore size Spherisorb ODS-2C_{18} reverse-phase column (4.6×250 mm). The mobile phase was 50 mmol/l of sodium acetate, 50 mmol/l of sodium citrate and 8% (v/v) methanol, pH 3.1 (corrected with HCl 12 N). HPLC analysis was performed under isocratic conditions at a flow rate of 1 ml/min and UV detector set at 274 nm. Tyr nitration levels were expressed as 3-NT (mmol/l)/Tyr (mmol/l) (N: 6/group).

2.3.3.4 Neurochemical Parameters

For the [^{3}H]glutamate uptake assay, the spinal cord tissue was used to prepare synaptosomes as described previously (NOGUEIRA et al., 2002). The synaptosomal preparation was washed twice by suspending in three volumes of 0.3 M sucrose, in 15 mM Tris-acetate buffer (pH 7.4), and centrifuging at 35000×g for 15 min. The final pellet was suspended in 0.3 M sucrose, 15mM Tris-acetate buffer (pH 7.4), and firstly incubated with Tris-HCl buffer (composition in mM Tris-HCl 27, NaCl 133, KCl 2.4, $MgSO_4$ 1.2, KH_2PO_4 1.2, Glucose 12, $CaCl_2$ 1.0) pH 7.4 (adjusted with HCl), for 10 min at 37 °C. The [^{3}H]glutamate uptake was then initiated by adding to the medium [^{3}H]glutamate (final concentration 100 mM) for 1 min at 37 °C. The reaction was stopped by centrifugation (16000×g, 1 min, 4 °C), and the pellets were washed thrice in Tris/HCl buffer. Radioactivity present in pellet was measured in a scintillation counter. Specific [^{3}H]glutamate uptake was calculated as the difference between the uptake obtained in the incubation medium described above, and the uptake obtained with a similar incubation medium in which NaCl was replaced by choline chloride. The values were expressed as pmol [^{3}H]glutamate/mg protein/min (N: 4/group).

Differently, for the Na^+, K^+-ATPase activity assay, the spinal cord tissue was homogenized in cold 50 mM Tris–HCl, pH 7.4 (1/5, w/v). The homogenate was centrifuged at 2400×g for 10 min to yield a pellet that was discarded and a low-speed supernatant (S_1). The reaction mixture for the assay contained 3 mM $MgCl_2$, 125 mM NaCl, 20 mM KCl, and 50 mM Tris-HCl, pH 7.4, in a final volume of 500 µl. For obtaining the ouabain-sensitive activity, S_1 (50 µl) was pre-incubated at 37 °C for 10 min in the reaction mixture with the presence or absence of ouabain (10 µl; final concentration 0.1 mM). The reaction was initiated by the addition of ATP to a final concentration of 3.0 mM and incubated at 37 °C for 30 min. Na^+, K^+-ATPase activity

was calculated by the difference between the two assays (presence or absence of ouabain). Released inorganic phosphate (Pi) was measured by the previously described method (FISKE e SUBBAROW, 1925). The values were expressed as nmol Pi/mg protein/h (N: 6/group).

2.3.3.5 Oxidative Stress Parameters

It was also investigated the following oxidative stress parameters: malondialdehyde (MDA) and non-protein thiol (NPSH) levels and the activities of Glutathione Peroxidase (GPx), Glutathione Reductase (GR) and Glutathione-S-Transferase (GST) (N: 6/group). For these assays, the spinal cord tissues were homogenized in cold 50 mM Tris–HCl, pH 7.4 (1/5, w/v), they were centrifuged at 2 400×g for 10 min to yield a low-speed supernatant (S_1).

HPLC procedure for MDA determination was done as described by Grotto *et al.* (2007) with slight modifications. MDA is an end-product of the peroxidation of lipids. MDA reacts with TBA to generate a colored product that can be measured optically at 532 nm (OHKAWA et al., 1979). Briefly, a volume of 75 µl of S_1 was added to 25 µl of NaOH 3 N and incubated at 60 °C for 30 min. After that, it was added 125 µl of H_3PO_4 6% and 100 µl of TBA 0.8% and the mixture was heated at 90 °C for 2h. Then the mixture was cooled and kept at -20 °C until extraction with n-butanol. For this, 50 µl of 10% SDS was added and the extraction with 300 µl of n-butanol was carried out by vortex-mixing for 1 min and centrifuged at 3 000×g for 10 min. The TBA-MDA adduct present in the butanol layer was analyzed on a Shimadzu® HPLC apparatus. The analytical column was 5 µm particles and 100 Å pore size, Phenomenex® ODS-2 C18 reverse-phase column (4.6×150 mm, Allcrom, BR). The mobile phase was a mixture of Milli-Q water and methanol (50:50; v/v).HPLC analysis was performed

under isocratic conditions at a flow rate of 0.6 ml/min and UV detector set at 532 nm, with a sample volume injection of 20 µl. The lipid peroxidation was expressed as µmol MDA/mg protein.

NPSH levels were determined by the method of Ellman (1959). To determine NPSH, S_1 was mixed (1:1) with 10% TCA. After the centrifugation, the protein pellet was discarded and free –SH groups were determined in the clear supernatant. An aliquot of supernatant was added in 1 M potassium phosphate buffer (pH 7.4) and 10 mM DTNB. The color reaction was measured at 412 nm. NPSH levels were expressed as Abs/g tissue.

GPx activity in S_1 was assayed spectrophotometrically by the method of Wendel (1981), through the GSH/NADPH/glutathione reductase system, by the dismutation of H_2O_2 at 340 nm. S_1 was added in GSH/NADPH/glutathione reductase system and the enzymatic reaction was initiated by adding H_2O_2. In this assay, the enzyme activity was indirectly measured by means of NADPH decay. H_2O_2 is reduced and generates GSSG from GSH. GSSG is regenerated back to GSH by glutathione reductase present in the assay media at the expenses of NADPH. The enzymatic activity was expressed as nmol NADPH/min/mg protein.

GR activity in S_1 was determined as described by Calberg and Mannervik (1985). In this assay, GSSG is reduced by GR at the expense of NADPH consumption, which was followed at 340 nm. GR activity is proportional to NADPH decay. The enzymatic activity was expressed as nmol NADPH/min/mg protein.

GST activity was assayed spectrophotometrically at 340 nm by the method of Habig*et al.*(1974). The reaction mixture contained an aliquot of S_1, 0.1 M potassium phosphate buffer (pH 7.4), 100 mM GSH and 100 mM CDNB, which was used as

substrate. The enzymatic activity was expressed as nmol CDNB conjugate/min/mg protein.

2.4 Protein Quantification

Protein concentration was measured by the method of Bradford (BRADFORD, 1976), using bovine serum albumin (1 mg/mL) as a standard.

2.5 Statistical Analysis

The results are presented as mean ± SEM. Data were analyzed using two-way analysis of variance (ANOVA) followed by the Newman-Keul's multiple range test when appropriate; for the behavioral tests, it was used two-way ANOVA of repeated measures followed by Bonferroni post-hoc analyses for the time versus treatment parameters as well as two-way ANOVA followed by the Newman-Keul's for the area under curve (AUC). p values less than 0.05 ($p < 0.05$) were considered as indicative of significance.

3 Results

3.1 Behavioral Assessment:

3.1.1 BPIS antinociceptive effect on the CFA-induced Inflammatory Pain Model

The mice receiving 20 µl CFA in the plantar surface developed mono-arthritis within 24 h. In order to measure inflammatory pain-related mechanical allodynia, this behavioral parameter was assessed with the von Frey test. As shown in Figure 2A, the percentage of response to VFH stimulation was increased by CFA injection, which was observed during all test assessment. The animals that received orally

BPIS at a dose of 1 mg/kg, but not at 0.1 mg/kg, demonstrated a reduction in mechanical allodynia induced by CFA. Statistically significant differences compared to the CFA group, in the animals that received BPIS at a dose of 1 mg/kg, were found starting 30 min after treatment and were maintained for all the investigated time (8h) [$F_{(30,210)}$ = 2.67; p < 0.001]. In addition, as seen in Figure 2B, the animals that received the positive control DEX at a dose of 2 mg/kg p.o. also had an antinociceptive effect starting 30 min after treatment and up to 8h [$F_{(18,140)}$ = 4.11; p < 0.001].

The two-way ANOVA of AUC demonstrated a significant interaction between CFA and BPIS, at a dose of 1 mg/kg [$F_{(1,20)}$ = 102.08; p < 0.001], but not at 0.1 mg/kg [p > 0.05]. Moreover, the two-way ANOVA of AUC data revealed a significant CFA and DEX interaction [$F_{(1,20)}$ = 32.624; p < 0.001].

3.1.2 Blockade of the antinociceptive effect of BPIS by L-arginine

Pre-treatment with L-arginine at a dose of 600 mg/kg (s.c.) abolished the antinociceptive effect of BPIS (1 mg/kg, p.o.) (Figure 3) during all the test assessment [$F_{(18,140)}$ = 4.59; p < 0.001]. Moreover, the two-way ANOVA of AUC data demonstrated a significant interaction between L-arginine and BPIS treatments [$F_{(1,20)}$ = 86.047; p < 0.001].

3.2 Tissue Analysis

3.2.1 Effect of BPIS on the MPO activity in the Paw and Spinal Cord Tissues

Related to the MPO activity in the paw tissue, the two-way analysis revealed a main effect of CFA i.pl. injection [$F_{(1,20)}$ = 41.56; p < 0.001], being observed that CFA increased the MPO activity in this tissue but BPIS did not reverse this alteration

(Fig. 4A). In the spinal cord tissue, the MPO activity was not detected in any studied group by the assayed method (data not shown).

3.2.2 Effect of BPIS on the NO_x levels in the Paw and Spinal Cord Tissues

In relation to the spinal cord tissue, the two-way analysis revealed an interaction between CFA and BPIS treatments [$F_{(1,20)} = 4.53$; $p < 0.05$] (Fig. 4B) and the post-hoc analysis demonstrated that the NOx levels were increased by CFA and BPIS diminished the increase in this parameter.

It was observed a main effect of CFA [$F_{(1,20)} = 4.43$; $p < 0.001$] in the NO_x levels in the paw tissues of the animals that received CFA i.pl. injection (Fig. 4C), represented by an increase in these levels induced by CFA.

3.2.3 Effect of BPIS on the 3-NT levels in the Paw and Spinal Cord Tissues

Regarding to the spinal cord 3-NT levels, the two-way analysis revealed neither main effect nor interaction between both treatments (Fig. 4D).

Similar to the observed in the previous parameters, it was observed a main effect of the CFA group [$F_{(1,20)} = 38.63$; $p < 0.001$]; CFA i.pl. injection induced an increase in 3-NT levels in the paw tissue (Fig. 4E).

3.3 Effect of CFA and BPIS on Neurochemical Parameters

Regarding the [^{3}H]glutamate uptake by synaptosomes in the spinal cord, the two-way ANOVA showed a significant interaction between CFA and BPIS treatments [$F_{(1,20)} = 22.78$; $p < 0.001$]. In addition, BPIS treatment was effective against the decrease in [^{3}H]glutamate uptake caused by the CFA i.pl. injection in mice (Table 1).

There was not a significant effect of CFA or BPIS in the Na^+, K^+-ATPase activity in spinal cord homogenates for any group (Table 1).

3.4 Effect of CFA and BPIS on Oxidative Stress Parameters

The results related to oxidative stress parameters are depicted in Table 2. The two-way ANOVA of MDA determination yielded a significant main effect of CFA i.pl. injection [$F_{(1,20)} = 5.20$; $p < 0.05$]. CFA significantly induced lipid peroxidation in spinal cord of mice and BPIS protected against the increase in these levels.

Regarding the NPSH levels in the spinal cord tissue, two-way ANOVA revealed a significant main effect of BPIS treatment [$F_{(1,20)} = 6.39$; $p < 0.05$] and a significant interaction between CFA and BPIS treatments [$F_{(1,20)} = 5.70$; $p < 0.05$]. However, post-hoc comparisons showed that only NPSH levels from animals that received both BPIS and CFA were different from all the other groups.

Two-way analysis demonstrated that there was no significant interaction between CFA and BPIS treatments for GPx, GR and GST activities in the spinal cord tissues of mice.

4 Discussion

The present study demonstrated that BPIS reversed against CFA-induced inflammatory pain model and this effect seems to be rather central than local because BPIS blocked alterations in spinal cord but not in paw tissues of mice. It was also demonstrated that pre-treatment with L-arginine blocked the antinociceptive effect of BPIS, suggesting that the L-arginine-NO pathway may be involved in its mechanism of action. Moreover, the results showed that BPIS: abolished the increase in NOx levels; and improved the reduction in glutamate uptake and rise in

MDA levels in spinal cord tissue when compared with those of the CFA group. Additionally, BPIS also induced a rise in NPSH levels in CFA-injected animals, which can be related to its antioxidant potential and could contribute to its anti-inflammatory action.

Administration of BPIS to mice, at a dose of 1 mg/kg but not at 0.1 mg/kg, was effective against induction of allodynia in the CFA model. The CFA i.pl. injection has been used as a rodent model of inflammatory nociception for exhibiting many features similar to arthritis, such as swelling and stiffness of joints and paws and development of symptoms like mechanical or thermal allodynia and hyperalgesia (NAGAKURA et al., 2003). The CFA injection leads to changes in the nociceptive pathways, those alterations can include sensitization of local nociceptors and central neurons (AOUAD et al., 2014). The release of several inflammatory and nociceptive mediators by primary sensory fibers can modify function of neurons and glial cells in the central nervous system (CNS), turning these neuronal cells more excitable and prompt to transmit the nociceptive signals to other sites (AOUAD et al., 2014; JI, R. R. e WOOLF, 2001).

During inflammatory process, the NO molecule is synthesized mainly via immune cells in the reaction catalyzed by the enzyme iNOS, in order to protect the organism against infections or injuries (COLEMAN, 2001). Nevertheless, when NO is overproduced, it acts as a pro-inflammatory molecule leading to activation of enzymatic cascades linked to cytokines production, cell differentiation and apoptosis (COLEMAN, 2001). Furthermore, high levels of NO can also undergo reaction with other molecules to produce other reactive species, for example nitroxyl, peroxynitrite, and S-nitrosothiols, contributing to the development of nitrosative and oxidative stress (KELM, 1999). L-arginine is used as substrate of iNOS, consequently its

administration enhances this pathway, increasing NO levels and downstream molecules such as cyclic GMP (EPSTEIN et al., 1993). When L-arginine was administered to mice before BPIS treatment, the protective effect of BPIS against CFA-induced mechanical allodynia was abolished, suggesting that this pathway is involved in the mechanism of action of BPIS.

In order to investigate tissue alterations induced by CFA that could be involved in the mechanism of action for BPIS antinociceptive effect, the spinal cord and the paw tissues were extracted. In the spinal cord tissue, BPIS reversed the NO_x levels, but not MPO activity or 3-NT levels, increased by CFA, reinforcing the participation of the L-arginine-NO pathway in the mechanism of BPIS antinociceptive effect. A previous study showed that BPIS can act as a NO_x scavenger *in vitro* (CHAGAS et al., 2015); in addition with the data observed in the current study, this could explain why BPIS has antinociceptive effect and inhibits the L-arginine-NO pathway. Regarding the paw tissue analysis, CFA induced changes in inflammatory mediators such as increase in MPO activity, NO_x and 3-NT levels. However, BPIS was not effective to reduce these alterations, at least in the dose responsible for the antinociceptive effect. These results seem to show that BPIS acts largely by central rather than local mechanisms. Accordingly, some reports regarding the organoselenium distribution point out to CNS as important target for pharmacological or even toxicological action of these compounds (BRÜNING et al., 2014; PRIGOL et al., 2012).

NO, besides a non-classical neurotransmitter, can also modulate other neurotransmitters release, uptake or signaling cascades (CALABRESE et al., 2007). As neurotransmission parameters, the Na^+, K^+-, ATPase activity and the glutamate uptake were also evaluated in the spinal cord tissue, whereas these mechanisms can

be altered in pathological pain processes (TAO et al., 2005; WANG et al., 2015). It has been reported that inflammatory processes can alter the activity of glutamate transporters, generally decreasing the glutamate uptake and leading to increased nociceptive behavior (ROSA et al., 2015; YASTER et al., 2011). Raju *et al.*(2015) reported that glutamate uptake, especially by the glutamate transporter 1, can be significantly reduced through s-nitrosylation of cysteine residues, and even the translocation of transporters to membrane surface can be affected. In our study, we observed a significant reduction of glutamate uptake by CFA, and BPIS in counterpart reestablished it to control levels, effect that could also be related to BPIS effect in the levels of NO-related compounds by CFA. It is well-known that Na^+, K^+-ATPase is the enzyme responsible for the active transport of sodium and potassium ions, required for vital functions, such as membrane cotransport and excitability (LEES, 1991). Although there are reports that Na^+, K^+-ATPase activity can be altered by CFA injection, no alteration was found in the present study (BOLDYREV et al., 1997; WANG et al., 2015).

Inflammatory nociceptive conditions can lead to oxidative stress with consequent formation of lipid peroxidation products and oxidation of thiols either locally or in the spinal cord (ROSSATO et al., 2010; ZAMBELLI et al., 2014). In the current study, the CFA injection in mice induced significant increase in MDA levels in the spinal cord and BPIS blocked this alteration. BPIS has been already studied as an antioxidant compound against oxidative stress in both *in vitro* and *in vivo* models (CHAGAS et al., 2015) and this property could contribute to decrease the lipid peroxidation induced by CFA. Moreover, despite the fact that CFA did not affect either the activity of the investigated antioxidant enzymes (GPx, GR and GST) or levels of NPSH, when the animals received both BPIS and CFA, NPSH levels were

increased. It is notorious that NPSH can be used to detoxify reactive species augmented in situations of oxidative stress (DAVIS et al., 2001). In a previous study with this compound, we demonstrated that BPIS can also mimic the activities of antioxidant enzymes such as GST and dehydroascorbate reductase *in vitro*, at the expense of glutathione to detoxify reactive species, and this might contribute to its *in vivo* antioxidant properties (CHAGAS et al., 2015).

5 Conclusions

Taken together, the present results indicate that BPIS might be of potential interest for the development of new strategies for treatment of inflammatory pain. The data show that BPIS acts predominantly via central mechanisms and its effect likely involves an interaction with the L-arginine-NO pathway and the antioxidant potential of the studied compound. Although the results are motivating, further results would be necessary for better understanding the complete mechanism by which this compound acts and its physiological effects in order to endorse the hypothesis of BPIS as an anti-inflammatory compound.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Tables

Table 1. Effect of CFA and BPIS (1 mg/kg, p.o.) on the neurochemical parameters in the spinal cord.

Group	[^{3}H]glutamate uptake	Na^+, K^+-ATPase activity
Control	8.13 ± 0.52	849.1 ± 103.8
BPIS	6.12 ± 0.89	738.8 ± 100.8
CFA	4.31 ± 0.55*	962.6 ± 171.4
CFA + BPIS	9.53 ± 0.97##	657.0 ± 87.4

Data are expressed as pmol [^{3}H]glutamate/mg protein/min (synaptosomal [^{3}H]glutamate uptake) and nmol Pi/mg protein/h (Na^+, K^+-ATPase activity). The results represent the means ± SEM of four or six animals for [^{3}H]glutamate uptake and Na^+, K^+-ATPase activity, respectively. The symbols denote significant difference *p<0.05 compared with the control group; ##p<0.01 compared with the CFA group; two-way ANOVA followed by the Newman-Keuls.

Table 2. Effect of CFA and BPIS (1 mg/kg, p.o.) on the oxidative stress parameters.

Group	MDA levels	NPSH levels	GPx activity	GR activity	GST activity
Control	9.13 ± 0.86	0.57 ± 0.041	1.72 ± 0.23	121.2 ± 1.5	306.1 ± 24.7
BPIS	9.43 ± 1.26	0.57 ± 0.037	2.12 ± 0.62	119.4 ± 4.9	293.5 ± 17.1
CFA	13.02 ± 1.00*	0.51 ± 0.024	2.13 ± 0.52	122.3 ± 8.2	268.8 ± 16.5
CFA + BPIS	9.89 ± 0.56#	0.72 ± 0.060@	2.16 ± 0.32	116.1 ± 11.3	313.0 ± 15.9

Data are expressed as µmol MDA/mg protein (MDA levels); Abs/mg protein (NPSH levels); µmol NADPH/mg protein/min (GPx and GR activities) and nmol CDNB conjugate/mg protein/min (GST activity). The results represent the means ± SEM of six animals. The symbols denote significant difference *p<0.05 compared with the

control group; #p<0.05 compared with the CFA group; @p<0.05 as compared to all the other groups; two-way ANOVA followed by the Newman-Keuls.

Figure legends

Figure 1. Chemical structure of BPIS.

Figure 2. Effect of BPIS (0.1 and 1 mg/kg; p.o.) (A) or DEX (2 mg/kg, p.o.) on mechanical allodynia in response to 10 applications of 0.6g VFH induced by CFA in the ipsilateral hindpaw. The mean of each animal was determined 24h after CFA-injection (0) and (0.5; 1; 2; 4 and 8h) subsequent to BPIS or DEX treatment. The results represent the means ± SEM of six animals. The symbols denote significant difference ***p<0.001 compared with the control group; **p<0.01 compared with the control group; *p<0.05 compared with the control group; ###p<0.001compared with the CFA group; two-way ANOVA of repeated measures followed by Bonferroni for the time versus treatment parameters and two-way ANOVA followed by Newman-Keul's for the area under curve (AUC).

Figure 3. Effect of pre-treatment with L-arginine (600 mg/kg; i.p.) 20 min before BPIS (1 mg/kg, p.o.) on the antinociceptive effect of the compound in the CFA-induced inflammatory pain model. The mean of each animal was determined 24h after CFA-injection (0) and (0.5; 1; 2; 4 and 8h) subsequent to BPIS treatment. The results represent the means ± SEM of six animals. The symbols denote significant difference ***p<0.001 compared with the CFA + BPIS group; two-way ANOVA of repeated measures followed by Bonferroni for the time versus treatment parameters and two-way ANOVA followed by Newman-Keul's for the area under curve (AUC).

Figure 4. Effect of CFA and BPIS (1 mg/kg, orally) on the MPO activity (A) in the paw tissue, NO_x (B and C) and 3-NT levels (D and E) in the spinal cord and paw tissues,

respectively. The results represent the means ± SEM of six animals. The symbols denote significant difference *p<0.05 compared with the control group; ##p<0.01 compared with the CFA group; two-way ANOVA followed by the Newman-Keuls.

Figures

Figure 1.

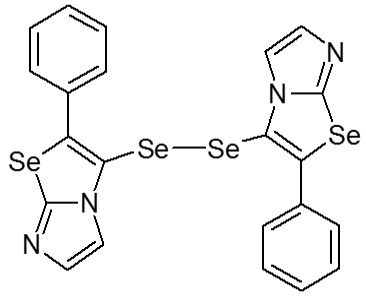


Figure 2.

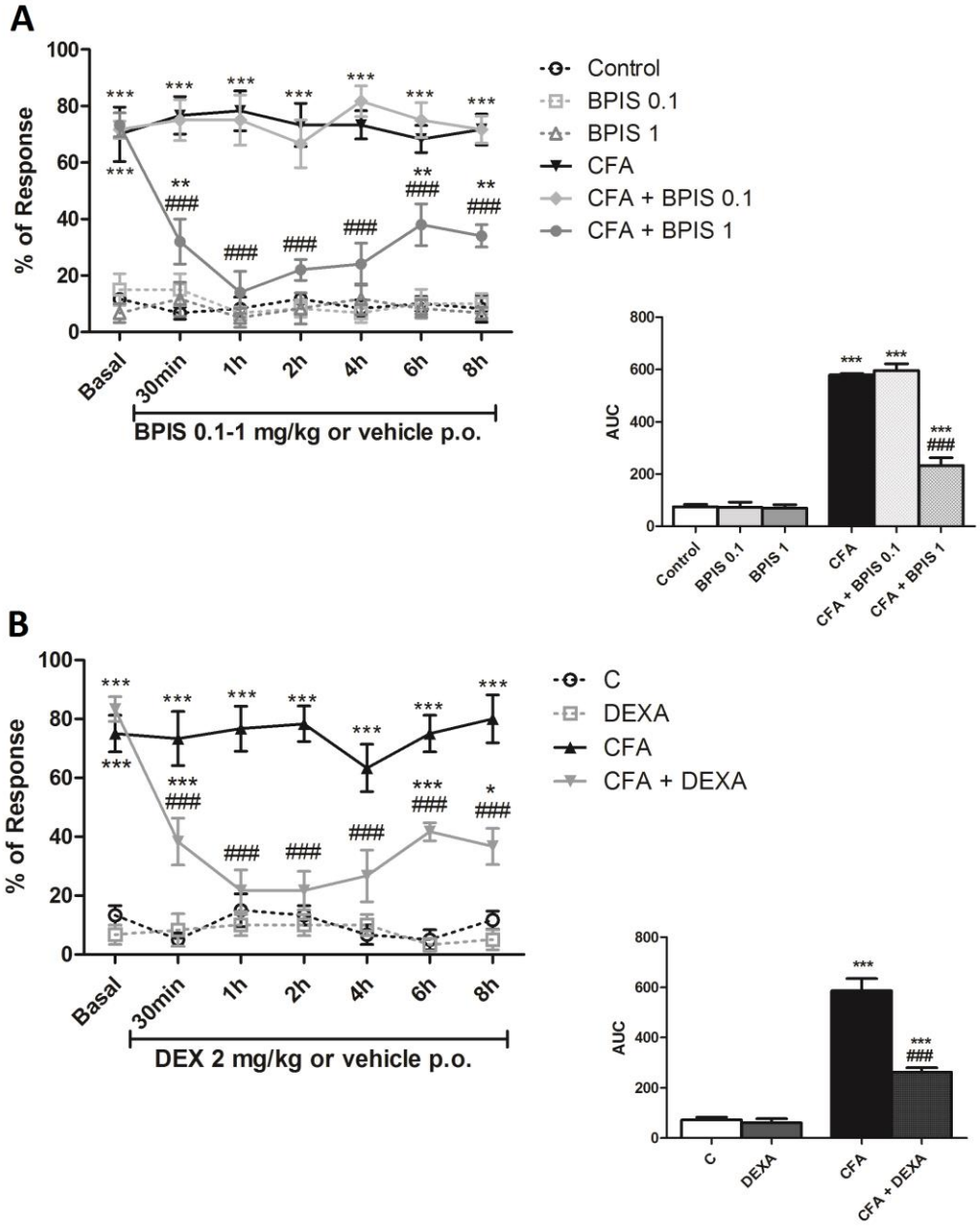
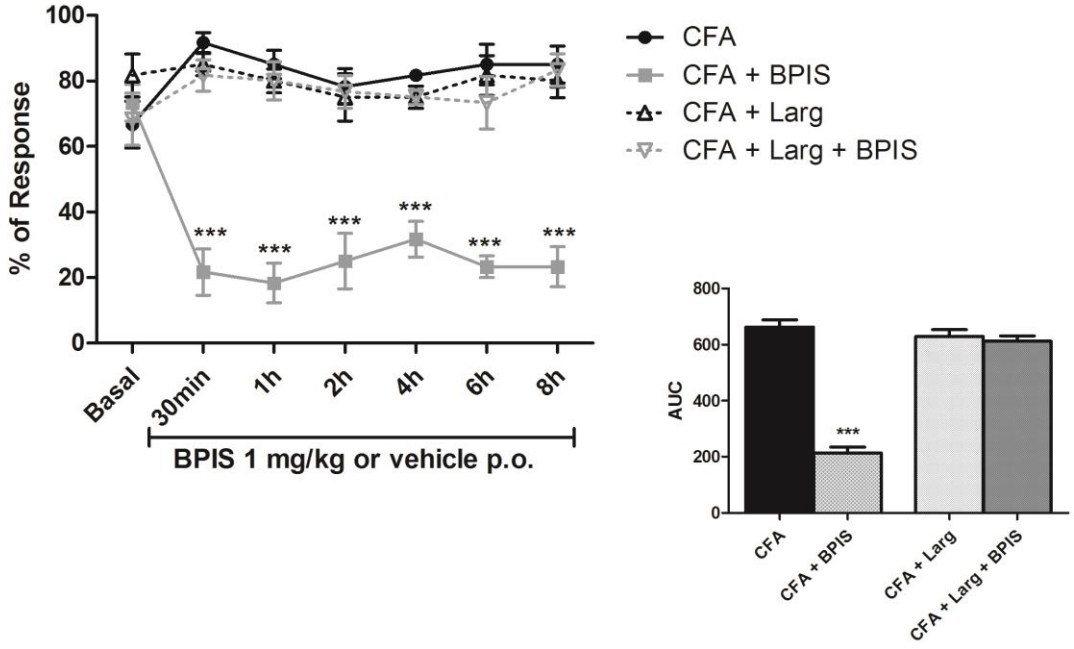


Figure 3.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2h
4h
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8h
BPIS 1 mg/kg or vehicle p.o.
CFA
CFA + BPIS
CFA + Larg
CFA + Larg + BPIS
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600
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AUC
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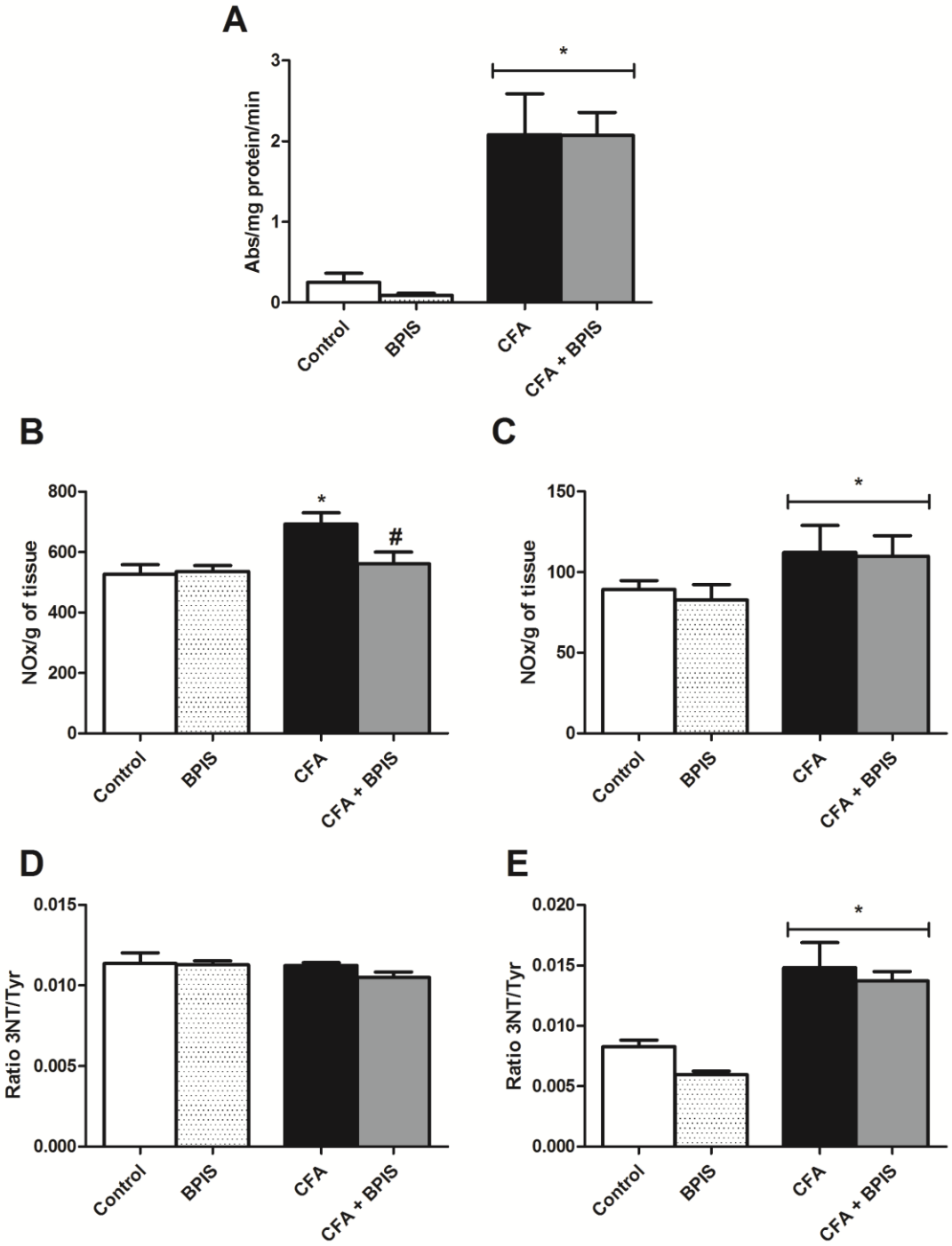
CFA
CFA + BPIS
CFA + Larg
CFA + Larg + BPIS

Figure 4.


A
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CFA + BPIS

3.4 Manuscrito 2

Disseleneto de Bis(fenilimidazoselenazolila) Exerce Efeito Antinociceptivo Através da Modulação da Atividade da Mieloperoxidase, Níveis de NOx e NFkB no Modelo de Artrite Induzida por Colágeno em Camundongos

BIS(PHENYLIMIDAZOSELENAZOLYL) DISELENIDE ELICITS ANTINOCICEPTIVE EFFECT BY MODULATING MYELOPEROXIDASE ACTIVITY, NOx AND NFkB LEVELS IN THE COLLAGEN-INDUCED ARTHRITIS MOUSE MODEL

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Bis(phenylimidazoselenazolyl) Diselenide Elicits Antinociceptive Effect by Modulating Myeloperoxidase activity, NOx and NFkB levels in the Collagen-induced Arthritis Mouse Model

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Abstract

Bis(phenylimidazoselenazolyl) diselenide (BPIS) is an organoselenium with acute antinociceptive and antioxidant properties. **Objectives**: The aim of the present study was to investigate BPIS effect on a collagen-induced arthritis (CIA) model in mice. **Methods:** Protocol of exposure consisted in arthritis-induction by chicken collagen-type II on day 0 with booster injection on day 21. On day 60 after collagen injection, incidence of mechanic allodynia (Von Frey test) or thermal hyperalgesia (Hot Plate test) was evaluated. During following 5 days, animals were treated with BPIS (0.1-1 mg/kg; p.o.; daily) or vehicle. On day 65, mice were killed and paws and spinal cord were removed for analyses. **Key findings:** Mice submitted to CIA model developed both mechanical allodynia and thermal hyperalgesia, which were reversed by BPIS at the highest dose. In paw, it was detected increase in myeloperoxidase activity in CIA group that was reversed by BPIS. In spinal cord, CIA group had increased NOx and NFkB levels and BPIS was effective to decrease these alterations. BPIS-treated animals had lower cyclooxygenase-2 levels in spinal cord. **Conclusions**: The myeloperoxidase activity in paw and NOx and NFkB levels in spinal cord are related to antinociceptive properties of BPIS in CIA model.

Keywords: organoselenium; collagen-induced arthritis; nociception.

1 Introduction

Rheumatoid arthritis (RA) is a progressive, systemic and immune-mediated disease. In addition, it is related to autoantibodies production, chronic inflammation and synovial swelling, which leads to cartilage destruction and joint damage (BEVAART et al., 2010). Joint pain is one of the most significant features of the RA disease and is present in patients with both active and minimal disease activity, and it is reported even in patients in remission (LEE, Y. C. et al., 2011; TAYLOR et al., 2010).

In order to study the pathogenic mechanisms of autoimmune arthritis as well as to evaluate potential new drugs or therapies, some animal models have been developed as research tools. The collagen-induced arthritis (CIA) in mice, one of the most commonly studied autoimmune models (BRAND et al., 2007) involves the immunization with an emulsion containing complete Freund's adjuvant and type-II collagen and share many similar features with the RA disease. This model is described by development joint inflammation and evocation of persistent pain-like behavior, being the last one related to spinal astrocytes and microglia activation (BAS et al., 2012).

Related to RA management, as the disease has not just one particular target to prevent tissue damage, it is difficult to find a drug that can normalize all the disease markers and prevent its progression. Even the most potent drugs, called disease-modifying antirheumatic drug (DMARDs), such as TNF inhibitors as well as purine and pyrimidine synthesis inhibitors, although efficient, they exhibit several concerns about side effects, mainly immunosuppression (BUER, 2015; SMOLEN e ALETAHA, 2015).

It is known that the oxidative stress, associated with cellular and tissue damage, is related to a wide range of diseases, and it has been linked as a detrimental consequence of chronic inflammation (VINCENT et al., 2002; WRUCK et al., 2011). Changes related to reactive oxygen and nitrogen species (ROS and RNS) are usually reversible and subtle, controlled by a cellular redox balance. In situations of extensive or persistent inflammation the regulatory redox systems are not enough efficient in order to counterbalance the evocation of toxic chemical events and the development of oxidative stress (CADENAS e DAVIES, 2000).

The interest in antioxidant molecules to treat inflammatory diseases has arisen in the last decades. Given that, studies have pointed out the anti-inflammatory

potential of selenium inorganic and organic compounds, in addition the antioxidant diaryl diselenides deserve special attention (BORTOLATTO et al., 2012; PRIGOL et al., 2009). Diphenyl diselenide $(PhSe)_2$ and its derivatives, for example, exerts anti-inflammatory effect both *in vitro* and *in vivo*, acting through nitrergic and glutamatergic pathways (SAVEGNAGO et al., 2007b; SHIN et al., 2009). Furthermore, the organoselenium bis(phenylimidazoselenazolyl) diselenide (Fig. 1), a compound that has antioxidant activity at low molecular range, as well as antinociceptive and anti-inflammatory potential in acute rodent models (CHAGAS et al., 2013; CHAGAS et al., 2015).

Regarding the above mentioned, the objectives of the current study were to evaluate: (I) the antinociceptive action of BPIS in the CIA model in mice; (II) possible nonspecific disturbances in the locomotor activity of mice treated with BPIS; and (III) the possible modulation of inflammatory parameters, for example myeloperoxidase (MPO) activity and NOx levels, in the spinal cord and paw tissue by CIA and BPIS treatment.

2 Material and Methods

2.1 Chemicals

BPIS was prepared and characterized according to Roehrs et al. (2012). Analysis of the ^{1}H NMR and ^{13}C NMR spectra shed analytical and spectroscopic data in full agreement with its assigned structure (ROEHRS et al., 2012). Complete Freund's Adjuvant (CFA), N, N, N', N'-tetramethylbenzidineand vanadium (III) chloride were purchased from the Sigma Chemical Co. (São Paulo, Brazil). Immunization Grade Chick Type II Collagen and Incomplete Freund's Adjuvant (IFA) were purchased from the Chondrex Inc. (Redmond, WA, USA). All other chemicals were of analytical grade and obtained from standard commercial suppliers. BPIS was dissolved in canola oil for the administration in the animals.

2.2 Animal

Male adult C57BL/6 mice (25-35g; 2-3 months old) from our own breeding colony were used for the experiments, due to specific features of its immune system related to the model (CAMPBELL et al., 2000). Animals were kept on a separate animal room, in a 12 h light/dark cycle, at a room temperature of 22 ± 2° C, with free

access to food (Guabi, RS, Brazil) and water. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Brazil (#066/2014).

2.3 Protocol for Induction of Collagen-Induced Arthritis (CIA) in Mice

For this model, immunization grade chick type II collagen (2 mg/ml, dissolved in acetic acid 0.05 M) was diluted in CFA (containing 1 mg/ml of inactive *Mycobacterium tuberculosis*; 1st injection) or IFA (2nd injection). The collagen was injected in a volume of 100 µl on day 0 (1st injection) and day 21 (2nd injection), according protocol of induction established by the manufacturer (Induction of arthritis with a booster injection - Chondrex Inc). The local of 1st intracaudal injection (2 cm to the base of the tail) and the 2nd injection (3 cm to the base of the tail) were different to avoid tissue necrosis. On day 60, the animals were evaluated for arthritis induction, on thermal hyperalgesia and mechanical allodynia. After this basal evaluation, on days 61 to 65, BPIS (0.1 or 1 mg/kg/day; p.o.) or vehicle was administered in order to examine the effect of the compound on this model. As a positive control, it was used dexamethasone (DEX; 0.25 mg/kg/day; i.p.) (KANG et al., 2000). The animals were divided in 8 groups (n=8 mice/group): Group I – Control animals treated with vehicle; Group II – CIA animals treated with vehicle; Groups III and IV – Control animals treated with BPIS 0.1 or 1 mg/kg/day, respectively; Groups V – Control animals treated with DEX; Groups VI and VII – CIA animals treated with BPIS 0.1 or 1 mg/kg/day, respectively; Group VIII – CIA animals treated with DEX.

2.4 Behavioral Assessment

2.4.1 Mechanical Allodynia

Mechanical allodynia was evaluated by the frequency of paw withdrawal response to ten applications (duration of 1-2 s each) of Von Frey hair filaments (Soelting, Chicago, IL, USA) under the hindpaw. A previous study indicated that the filament of 0.6g intensity produces an average withdrawal response of approximately 20% in control animals, an intermediate value for mechanical allodynia evaluation (VILAR et al., 2013). The test was evaluated on day 60 in order to verify the induction of mechanical allodynia in the arthritis model and after that, every two days (days 61, 63 and 65) the allodynia assessment was performed again in order to evaluate the effect of BPIS.

2.4.2 Locomotor Activity

The open-field was made of plywood and surrounded by walls 30 cm in height. The floor of the open-field, 45 cm in length and 45 cm in width, was divided by masking tape markers into 9 squares (3 rows of 3).Each animal was placed individually at the center of the apparatus and observed for 6 min to record the number of segments crossed with the four paws and the number of time rearing on the hind limbs (WALSH e CUMMINS, 1976). This behavioral test was performed on days 60, 61, 63 e 65, after the mechanical allodynia assessment.

2.4.3 Thermal Hyperalgesia

For the evaluation of the development of thermal hyperalgesia, the animals were submitted to the hot plate test. The mice were placed in an acrylic cylinder (20 cm in diameter) on the hot plate apparatus, maintained at 52 ± 1 °C (GARCIA-MARTINEZ et al., 2002; SAVEGNAGO et al., 2007a). The time (s) between the placement on the heated surface and licking of their hind paws or jumping was recorded as the response latency. A 60 s cut-off was used to prevent tissue damage. Similar to the mechanical allodynia assessment, this behavioral test was performed on days 60, 61, 63 e 65, after the locomotor activity assessment.

2.5 Tissue Analysis

Only the highest dose of BPIS (1 mg/kg, p.o.) was selected for the tissue analysis. The animals were killed and samples of hind paw and spinal cord tissue were rapidly dissected, weighed and placed on ice. These samples were removed for myeloperoxidase (MPO) activity, nitrate/nitrite (NOx) levels determination. In addition, the spinal cord tissue was also extracted for Western Blot analysis.

2.5.1 Myeloperoxidase Assay

The MPO activity was assayed according to the method of Suzuki *et al.* (1983) with some modifications. The spinal cord and the hindpaw tissue were homogenized in potassium phosphate buffer (20 mmol/l, pH 7.4; 1:5, w/v) containing ethylenediaminetetraacetic acid (0.1 mmol/l). After the homogenization, the samples were centrifuged at 2000×g at 4 °C for 10 min to yield a low-speed supernatant fraction (S_1). Then, the S_1 fraction was centrifuged again at 20000×g at 4 °C for 15

min to yield a final pellet (P_2) that was resuspended in medium containing potassium phosphate buffer (50 mmol/l, pH 6.0) and hexadecyltrimethyl ammonium bromide (0.5%). The samples were finally frozen and thawed three times for the posterior enzymatic assay.

For the MPO activity measurement, an aliquot of resuspended P_2 (100 µl) was added to a cuvette containing the medium of resuspension and N, N, N', N'-tetramethylbenzidine (1.5 mmol/l). The kinetic analysis of MPO was started after H_2O_2 (0.01%) addition, and the color reaction was measured at 655 nm at 37 °C. Results are expressed as absorbance (Abs)/mg protein/min (N: 6/group).

2.5.1 Determination of NOx content

The hindpaw and the spinal cord tissues were homogenized with $ZnSO_4$ (200 mM; 500 µl) and acetonitrile (96%; 500 µl), centrifuged at 16000×g at 4 °C for 30 min, and the supernatant was collected for assay of the nitrite plus nitrate content (MIRANDA et al., 2001). NOx content was estimated in a medium containing 300 µl of 2% VCl_3 (in 5% HCl), 200 µl of 0.1% N-(1-naphthyl) ethylene-diaminedihydrochloride, and 200 µl of 2% sulfanilamide (in 5% HCl). After incubating at 37 °C for 60 min, nitrite levels were determined spectrophotometrically at 540 nm, based on NOx/g of tissue (N: 6/group).

2.5.3 Western Blot Analysis

The spinal cord tissue was homogenized in a 5% SDS solution containing a cocktail of protease and phosphatase inhibitors (Sigma, São Paulo, SP, Brazil). The protein content was determined using the bicinchoninic acid assay (BCA) (Sigma, São Paulo, SP, Brazil). The sample extracts were diluted to a final protein concentration of 2 µg/µl in SDS solution and the amount of protein applied for SDS–PAGE analysis was 40 µg. The proteins, together with pre-stained molecular weight standards (Bio-Rad, São Paulo, Brazil), were applied to a 10% SDS–PAGE running gel with a 4% concentrating gel. After electro-transfer, membranes were blocked with Tris-buffered saline containing 0.1% Tween-20 and 3% bovine serum albumin (BSA) during 1 h. The nitrocellulose membranes (Amersham, SP, Brazil) were then incubated overnight at 4 °C with rabbit anti-COX-2 antibody (1:1000; Cell Signaling Technology, Beverly, MA, USA), rabbit anti-NFκB antibody (1:2000; Cell Signaling Technology, Beverly, MA, USA). The membranes were washed and incubated with

horseradish peroxidase-conjugated secondary antibodies for 1h at room temperature and developed with ECL kit (Termo-Fisher Scientific, Waltham, MA, USA). Densitometric analyses were performed using the NIH ImageJ software. After stripping, beta-actin was quantified as a loading control using a mouse anti-actin antibody (1:1000; Cell Signaling Technology, Beverly, MA, USA) as described above.

2.6 Protein Quantification

Protein concentration was measured by the method of Bradford (BRADFORD, 1976), using bovine serum albumin (1 mg/mL) as the standard.

2.7 Statistical Analysis

The results are presented as mean ± SEM. Data were analyzed using two-way analysis of variance (ANOVA) followed by the Duncan multiple range test when appropriate; for the behavioral tests, it was used two-way ANOVA of repeated measures followed by Bonferroni post-hoc analyses for the time versus treatment parameters as well as two-way ANOVA followed by the Newman-Keul's for the area under curve (AUC). The *p* values less than 0.05 ($p < 0.05$) were considered as indicative of significance.

3 Results

3.1 Behavioral Assessment

3.1.1 BPIS antiallodynic effect on the CIA Model

The two-way ANOVA of the mechanical allodynia behavioral data (time vs treatment) showed a significant treatment and time interaction [$F_{(15,168)} = 3.22$; $p < 0.001$] (Fig. 2). The post-hoc analysis revealed that in the baseline of mechanical allodynia taken on day 60, the animals submitted to the CIA model exhibited marked increase in the response to the VFH of 0.6 g. In addition, BPIS treatment at both doses (0.1 and 1 mg/kg) was effective in reducing this augment in response, since day 61 (1st day of treatment) to the end of treatment.

Regarding the AUC data, the two-way ANOVA showed a significant interaction between CIA and BPIS treatment [$F_{(1,28)} = 20.64$; $p < 0.001$ and $F_{(1,28)} = 34.06$; $p < 0.001$, respectively].

3.1.2 BPIS anti-hyperalgesic effect on the CIA Model

The two-way ANOVA of the thermal hyperalgesia behavioral data (time vs. treatment) revealed a significant treatment and time interaction [$F_{(15,168)}$ = 2.34; p < 0.001] (Fig. 3). The post-hoc analysis showed that the animals that received the collagen injection, presented on day 60 a decreased latency to response on the hot plate test. Moreover, BPIS treatment at the dose of 1 mg/kg was effective to increase this latency since the 1st day of treatment to the end of experiment, but the dose of 0.1 mg/kg was effective only when the test was performed on the 3rd day of treatment.

The two-way ANOVA of AUC data yielded a significant interaction between CIA and BPIS treatment with the dose of 1 mg/kg but not 0.1 mg/kg [$F_{(1,28)}$ = 23.87; p < 0.001 and $F_{(1,28)}$ = 1.89; p = 0.18, respectively].

3.1.3 Neither BPIS nor CIA model affects locomotor activity

The two-way ANOVA for both crossing and rearing numbers [$F_{(15,168)}$ = 0.65; p =0.827 and $F_{(15,168)}$ = 0.28; p = 0.997, respectively] did not revealed any interaction between treatments along the time (Fig. 4A and 4B).

Moreover, the two-way ANOVA for the AUC data revealed a non-significant CIA and BPIS interaction, neither at the dose of 0.1 nor at 1 mg/kg [$F_{(1,28)}$ = 0.14; p =0.713 and $F_{(1,28)}$ = 0.17; p = 0.679, respectively]. Similar was observed for the AUC data related to the rearing number, no interaction between CIA and BPIS treatment at the dose 0.1 nor 1 mg/kg [$F_{(1,28)}$ = 1.09; p =0.306 and $F_{(1,28)}$ = 0.67; p = 0.420, respectively].

3.2 Tissue Analysis

3.2.1 BPIS reversed the decrease in MPO activity induced by the CIA model in the paw but not in the spinal cord tissue

Fig. 5A shows the MPO activity in the paw tissue. The two-way ANOVA demonstrated significant main effects of CIA [$F_{(1,28)}$ = 6.92; p < 0.05] and BPIS treatment [$F_{(1,28)}$ = 15.08; p < 0.001]. The CIA model induced an increase in the activity of the enzyme and BPIS was effective to reverse this alteration. In the spinal cord tissue, the MPO activity was not detected in any studied group by the assayed method (data not shown).

3.2.2 BPIS reversed the increase in NOx levels induced by the CIA model in the spinal cord but not in the paw tissue

The two-way ANOVA of NOx levels in the spinal cord showed a significant interaction between CIA and BPIS [$F_{(1,28)}$ = 6.04; p < 0.05]. Post-hoc analysis revealed that CIA mice presented an increase in spinal levels of NOx and BPIS was effective to reverse this parameter to the control levels (Fig. 5B). Regarding the NOx levels in the paw tissue, the two-way ANOVA did not reveal a statistically significant interaction between CIA and BPIS [$F_{(1,28)}$ = 3.72; p = 0.064].

3.2.3 BPIS was effective against the increase in NFKB levels induced by the CIA model in the spinal cord

The results related to western-blot analysis are depicted in Fig. 6A. Two-way ANOVA of NFkB protein levels yielded significant main effects of CIA and BPIS treatment [$F_{(1,12)}$ = 16.78; p <0.01 and $F_{(1,12)}$ = 12.48; p < 0.01, respectively]. The CIA induced an increase in the NFkB levels, and BPIS treatment reduced these levels. Furthermore, control animals that received BPIS treatment had *per se* a decrease in the NFkB protein levels.

3.2.4 BPIS reduced COX-2 levels in the spinal cord

The two-way ANOVA of COX-2 protein levels revealed a significant main effect of BPIS treatment [$F_{(1,12)}$ = 8.41; p <0.05]. The levels of COX-2 enzyme were reduced in control and CIA experimental groups that received treatment with BPIS (Figure 6B).

4 Discussion

The findings of the present study indicate that the CIA model caused the development of hyperalgesia and allodynia in mice and these behavioral alterations were reversed by the BPIS treatment, without locomotor activity interference. In addition, BPIS also reversed the increase alterations in inflammatory tissue parameters in the CIA model, for example: BPIS decreased the augmented MPO activity in the paw tissue, as well as reversed the increase in NOx and NFκB levels in the spinal cord. Moreover, BPIS treatment *per se* decreased COX-2 and NFκB levels in the spinal cord tissue.

Joint pain is one of the most outstanding characteristics of RA; it can lead to psychological distress and quality of life impairment, being a cause of loss of joint mobility and proper function (MCDOUGALL, 2006). Because pain can not be measured directly in animals, allodynia and hyperalgesia in animals can be used as surrogates for assessment of enhanced nociception in models of pain-related diseases (SANDKUHLER, 2009). In the present study, we observed the mice that received tail injection of chicken collagen type II developed both mechanical allodynia and thermal hyperalgesia. The CIA group presented an increase in paw withdrawal responses after Von Fray hair stimulation, a possible indicator of mechanical allodynia (MALMBERG e CHAPLAN, 2002) subsequent to arthritis development. Furthermore, the CIA group also had diminished latencies to response in the Hot Plate test, pointing also to thermal hyperalgesia (MALMBERG e CHAPLAN, 2002). BPIS, in counterpart, was effective to reverse these behavioral alterations induced by the CIA model, corroborating with its antinociceptive potential. BPIS, when evaluated in acute models of thermal and chemical nociception, showed antinociceptive properties in mice, but at higher doses than that of observed in this protocol (higher than 10 mg/kg), and this could be related to the treatment schedule (in a single dose) or the type of induction (CHAGAS et al., 2013).

We also investigated possible interferences of BPIS treatment or the CIA model in the spontaneous exploratory behavior, assessed in the Open-Field Test. It is known that changes in locomotor activity can cause false-positive responses in nociceptive tests (LE BARS et al., 2001), then the lack of differences between the groups could exclude this interference in the previous mentioned tests. Only a difference along the days was observed in all experimental groups, being this a particularity of the test that is generally related to memory acquisition processes of the animal (BOLIVAR et al., 2000); however, even in the last day, the exploratory behavior was not abolished and all groups had the same sort of behavior.

Both paw and spinal cord tissues were collected for dosage of MPO activity and NOx levels. Regarding to the MPO, whose activity was only detectable in the paw tissue, this parameter was increased in the animals of the CIA group and animals that received BPIS treatment had this activity restored. It is known that the MPO is an enzyme present in the granules of leucocytes, mainly neutrophils and macrophages and responsible for secreting hypochlorite, being considered also an inflammatory process marker (BLANK et al., 2000). The CIA model shares some

similar features to the RA disease, being joint cell infiltration one of them, which could explain the increase in the MPO activity, observed in the paw tissue (BRAND et al., 2007; CAMPBELL et al., 2000). Moreover, the NO molecule is also recognized as a mediator of the physiological responses by multiple mechanisms, including the intracellular cascades, protein nitration and formation of other intermediaries (COLEMAN, 2001; RAJU et al., 2015). These intermediaries, including nitrite and nitrate, are formed via NO oxidation, especially when this molecule is overproduced in inflammatory situations (COLEMAN, 2001; KELM, 1999). Interestingly, in the present study, it was only observed an augment in this parameter by the collagen injection when analyzed in the spinal cord, but not in the paw tissue. Studies demonstrated the influence of peripheral inflammation in the activation of neuroimmune mechanisms and sensitization of pathways related to hyperalgesia and allodynia (JI et al., 2003; THOMSON et al., 2014). We have also to highlight that the CNS is an important target for the pharmacological and even toxicological effects of organoselenium compounds (BRÜNING et al., 2014; PRIGOL et al., 2012).

Regarding the above mentioned, the importance of the CNS for the pharmacological action of organoselenium compounds and the role of central mechanisms in the hyperalgesia and allodynia development, we investigated the effect of the CIA model and BPIS treatment in the levels of proteins such as NFkB and COX-2 in the spinal cord. NFkB is a transcription factor, required to induce the expression of several inflammatory and immune responses, which can be activated by a variety of pathogenic stimuli, including cytokines, growth factors and oxidative stress (MAKAROV, 2001). It is known that NFkB can be activated in the spinal cord in order to contribute to inflammatory pain hypersensitivity (LEE, K. M. et al., 2004). In the present study, the animals of the CIA group had higher levels of this factor and BPIS was not only effective to reduce the NFkB in the animals that received the collagen injection, but also in the control animals. BPIS could be interfering in the route of synthesis or activation of NFkB, similar to what was already reported for other diselenides *in vitro* (SHIN et al., 2009)

The CIA model has been widely used both to find some mechanisms related to RA-associated autoimmunity and for the study of potential and already-in-use anti-inflammatory drugs (BRAND et al., 2007; KANG et al., 2000). This model has some similar pathological features with RA, including synovial hyperplasia, joint cell infiltration and the nociceptive hypersensitivity (BRAND et al., 2007). The effect of

BPIS corroborates with its potential as an antinociceptive and anti-inflammatory agent, being this model of particular importance, because it is a model of a chronic disease.

5 Conclusions

In conclusion, the present data indicate that MPO activity in the paw tissue and spinal cord NOx and NFkB levels are related to the antinociceptive properties of BPIS in the CIA mouse model. Together, the current data can indicate the potential importance of BPIS for the development of novel clinically relevant anti-inflammatory drugs that could be used as an alternative for RA relief, as well as other inflammatory diseases.

Conflict of interest statement

The authors declare they have no conflict of interest.

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Figure Legends

Figure 1. Chemical structure of BPIS.

Figure 2. Effect of BPIS (0.1 and 1 mg/kg; p.o.) on mechanical allodynia induced by the CIA model in response to 10 applications of 0.6g VFH in each hindpaw. The mean of each animal was determined in the days 60, 61, 63 and 65. The results represent the means ± SEM of eight animals. The symbols denote significant difference ***p<0.001 compared with the control group; ###p<0.001compared with the CIA group; two-way ANOVA of repeated measures followed by Bonferroni for the time versus treatment parameters and two-way ANOVA followed by Duncan for the area under curve (AUC).

Figure 3. Effect of BPIS (0.1 and 1 mg/kg; p.o.) on thermal hyperalgesia induced by the CIA model in the hot plate test (52 ºC). The behavioural assesment was performed in the days 60, 61, 63 and 65. The results represent the means ± SEM of eight animals. The symbols denote significant difference ***p<0.001 compared with the control group; #p<0.05 compared with the CIA group; ##p<0.01 compared with the CIA group; ###p<0.001compared with the CIA group; two-way ANOVA of repeated measures followed by Bonferroni for the time versus treatment parameters and two-way ANOVA followed by Duncan for the area under curve (AUC).

Figure 4. Effect of BPIS (0.1 and 1 mg/kg; p.o.) and the CIA model on crossing (A) and rearing (B) numbers in the open-field test (6 min). The behavioral assessment was performed in the days 60, 61, 63 and 65. The results represent the means ± SEM of eight animals. Two-way ANOVA of repeated measures followed by Bonferroni for the time versus treatment parameters and two-way ANOVA followed by Duncan for the area under curve (AUC).

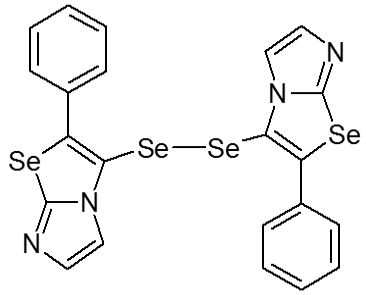
Figure 5. Effect of CIA model and BPIS (1 mg/kg, p.o.) on the MPO activity (A) in the paw tissue, NOx (B and C) in the spinal cord and paw tissues, respectively. The

results represent the means ± SEM of eight animals. The symbols denote significant difference *p<0.05 compared with the control group; ***p<0.001 compared with the control group; #p<0.05 compared with the CIA group; ###p<0.001 compared with the CIA group; two-way ANOVA followed by the Duncan.

Figure 6. Effect of CIA model and BPIS (1 mg/kg, p.o.) on the Western Blot analysis of the NF-κB (A) and COX-2 (B) in the spinal cord. The results represent the means ± SEM of four animals and the data are expressed ratio of the protein/actin. The symbols denote significant difference *p<0.05 compared with the control group; ***p<0.001 compared with the control group; ###p<0.001compared with the CFA group; $$$p<0.001 as compared to all the other groups; two-way ANOVA followed by the Duncan.

Figures

Figure 1.



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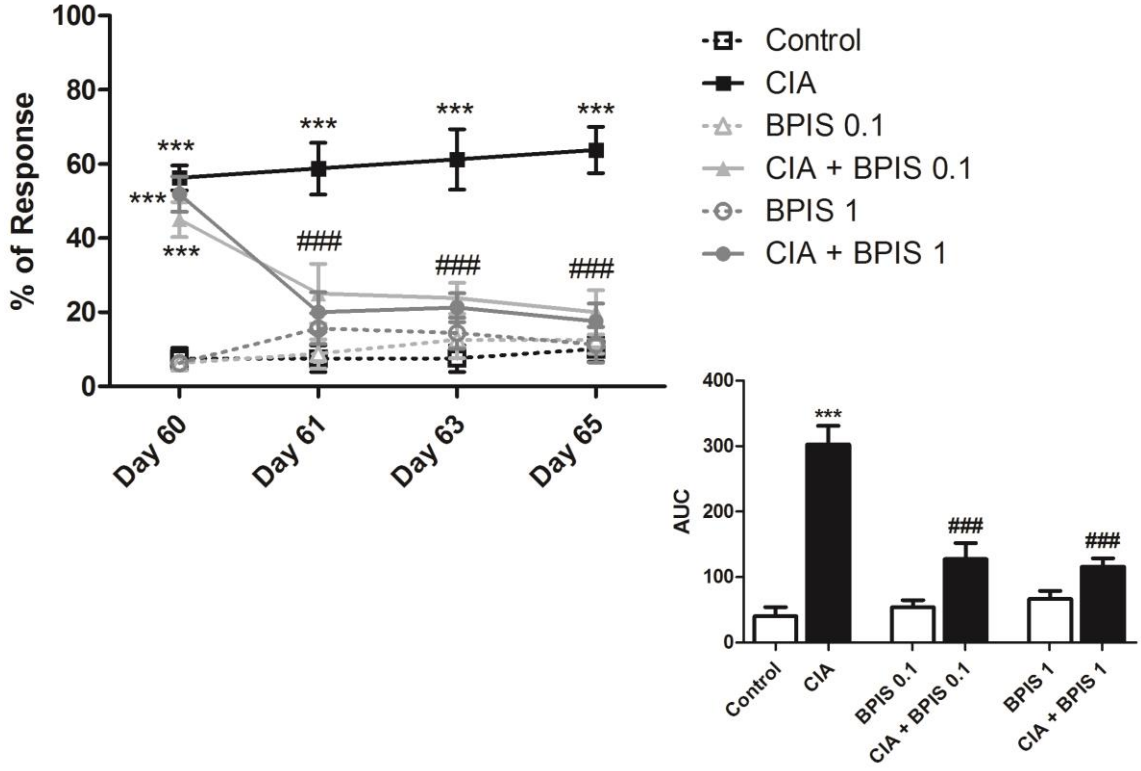

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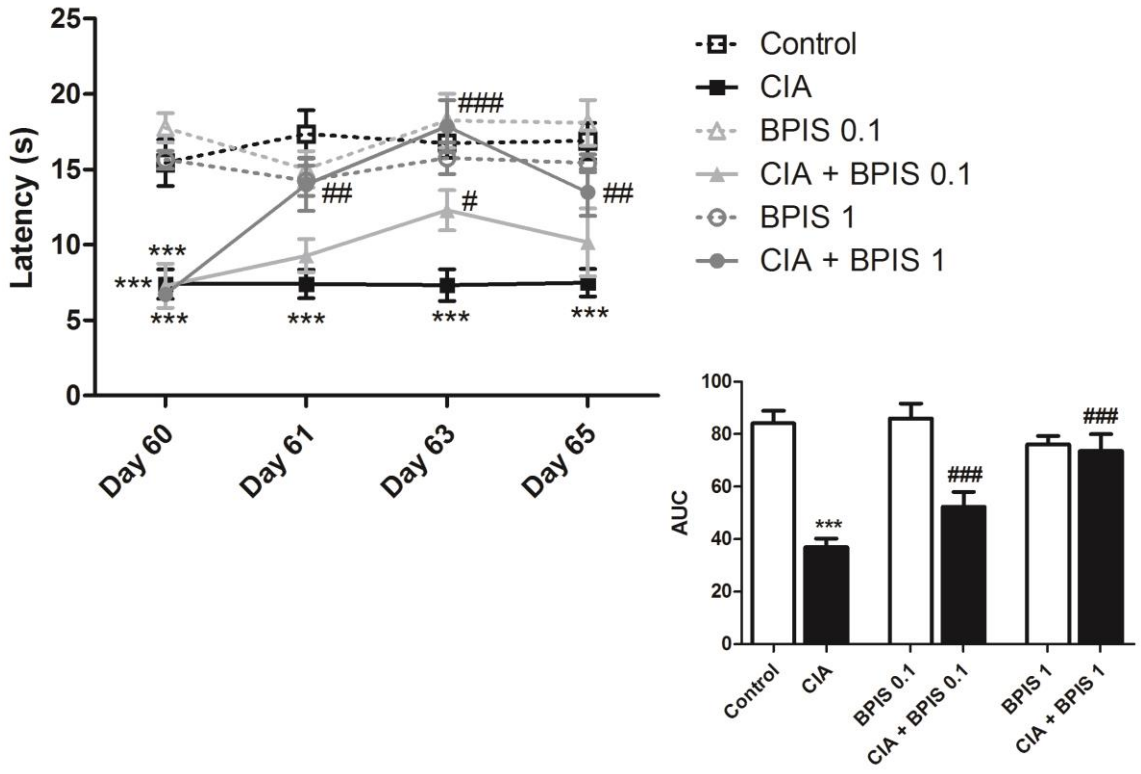

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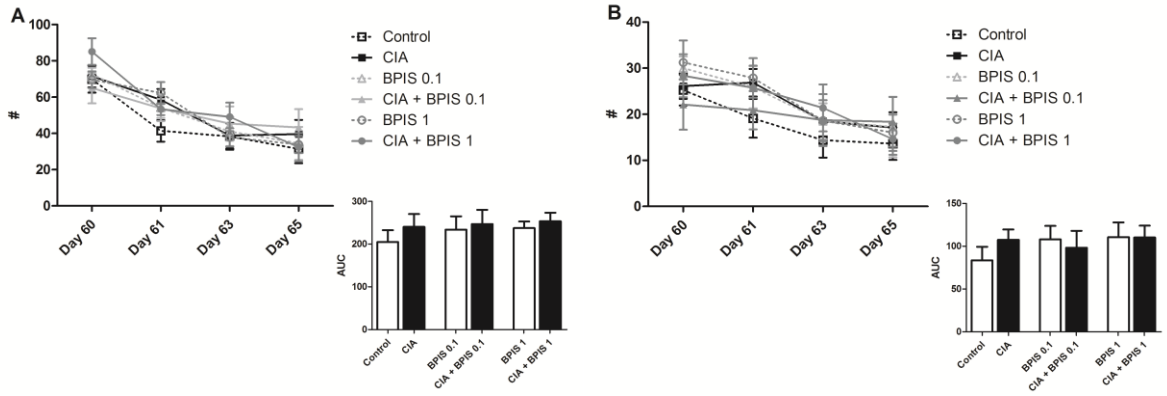

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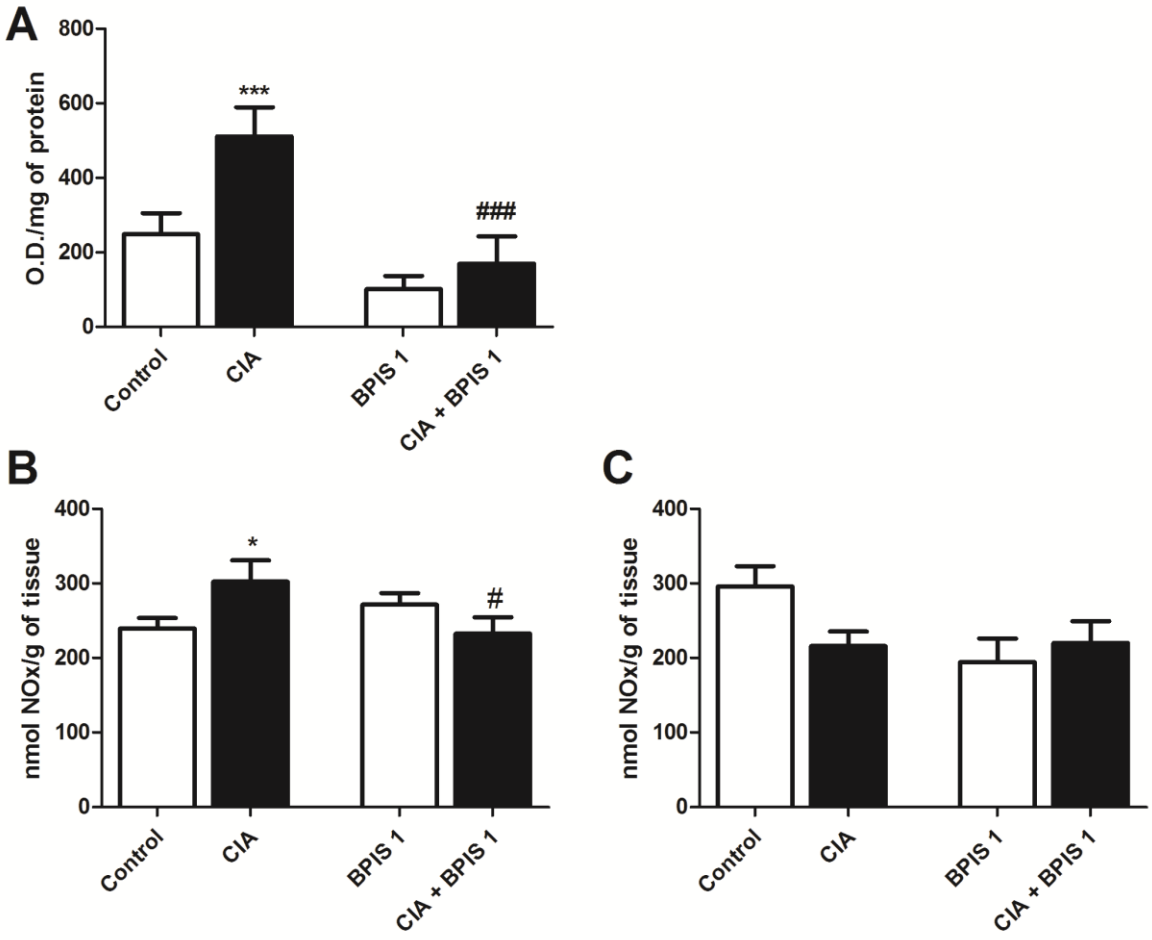
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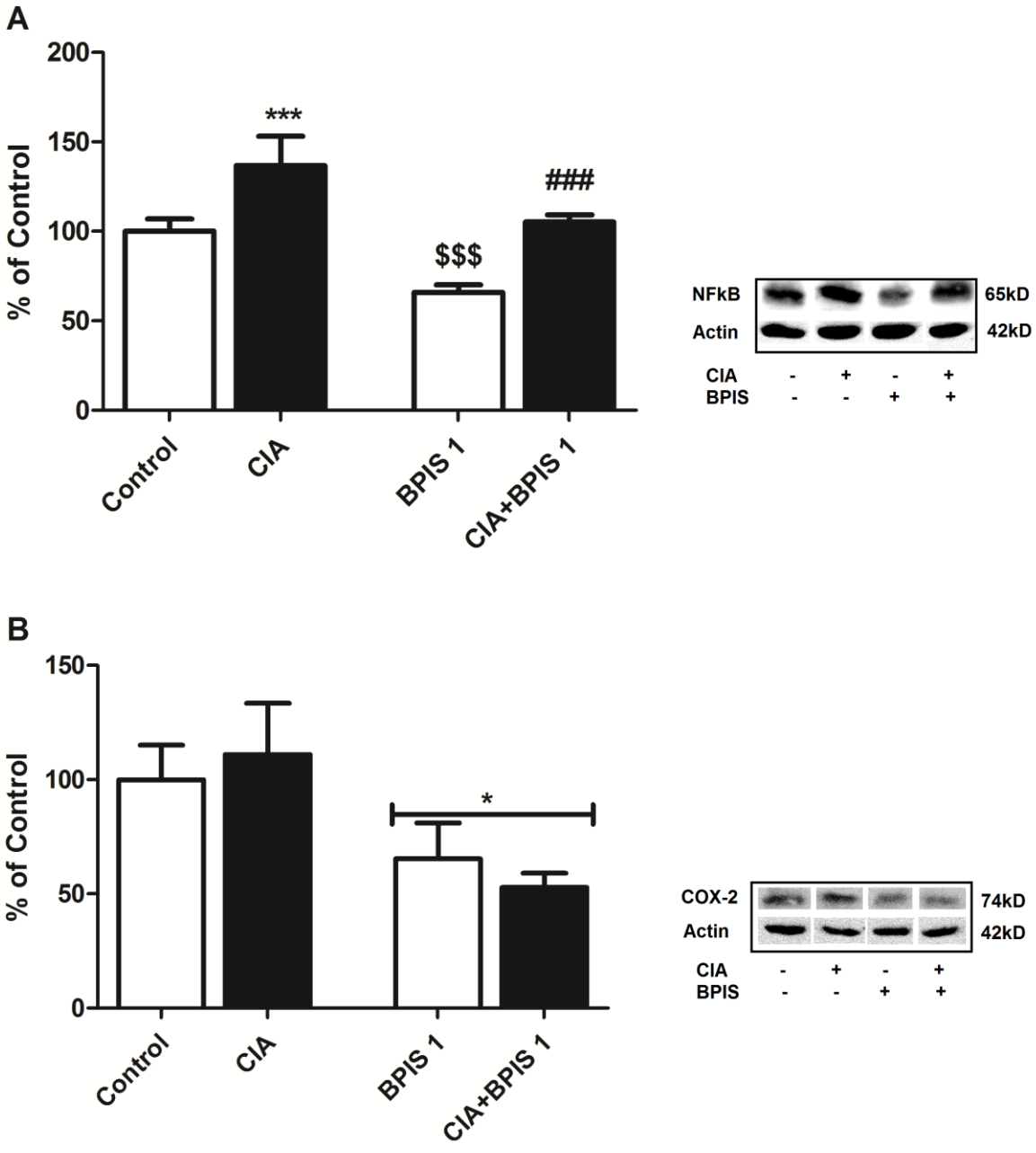
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4 DISCUSSÃO

O Se é um elemento-traço essencial para a saúde humana, principalmente devido a seu papel como componente de selenoenzimas, das quais um dos principais exemplo cita-se a GPx, desempenhando uma função importante em diversas atividades celulares, principalmente a manutenção do potencial redox intracelular (RAYMAN, 2000). Além disso, os compostos de selênio são geralmente divididos em duas formas: inorgânica e orgânica, sendo a forma orgânica reportada como mais biodisponível e menos tóxica (KIM e MAHAN, 2001; YOUNG et al., 1982). Os compostos orgânicos de selênio, com ênfase nas moléculas sintéticas, têm sido estudados nas últimas décadas, principalmente devido ao seu papel como moléculas antioxidantes (NOGUEIRA e ROCHA, 2011). Dentre os principais compostos orgânicos de selênio sintéticos antioxidantes, podemos citar o ebselen, o $(PhSe)_2$ e seus derivados, os quais tem inúmeras atividades farmacológicas e biológicas reportadas, incluindo importantes propriedades anti-inflamatórias e antinociceptivas (BRUNING et al., 2010; NOGUEIRA et al., 2003; PINTO et al., 2008; SCHEWE, 1995; SHIN et al., 2009).

Na busca por moléculas com potencial tanto antioxidante quanto antinociceptivo, esta tese focou seus estudos em um novo derivado dos disselenetos de diarila, a molécula do DFIS. Inicialmente, os experimentos dos **artigos 1** e **2**, foram feitos separadamente, com o objetivo de investigar as propriedades antinociceptivas e antioxidantes do DFIS, respectivamente, como uma triagem de seu potencial farmacológico. Em ambos os artigos também foi avaliado seu potencial toxicológico, através de experimentos tanto *in vitro* quanto *in vivo*. Além disso, no **manuscrito 1**, investigou-se o potencial antinociceptivo do DFIS em um modelo agudo de nocicepção de cunho inflamatório, o qual foi reforçado pelo seu estudo em um modelo de artrite reumatoide crônica, estudado no **manuscrito 2**.

No **artigo 1**, investigou-se o efeito antinociceptivo do DFIS frente a modelos de nocicepção térmica e química, assim como possíveis alterações em parâmetros locomotores ou toxicológicos. Primeiramente, o DFIS foi avaliado no teste de nocicepção térmica de imersão da cauda, com o intuito tanto de avaliar a curva de tempo em que o composto poderia apresentar seu efeito antinociceptivo, assim como a possível faixa de dose. A partir do teste de Imersão da Cauda, observou-se que a administração oral do DFIS (50 mg/kg) aumentou o delta (Δ) de latência para retirada da cauda desde 15 min até 4h após o tratamento, e também foi analisado

que o composto apresentava seu melhor efeito no tempo de 30 min. Sendo assim, para todos os outros testes de nocicepção foi escolhido o tempo de 30 min para tempo de pré-administração antes da realização dos procedimentos. Na curva de dose dos testes térmicos observou-se efeito antinociceptivo do BPIS nas doses de 25, 50 e 100 mg/kg, embora a dose de 100 mg/kg não apresentou aumento no efeito quando comparada a dose de 50 m/kg. A principal diferença entre estes dois testes térmicos é que o teste de imersão da cauda está relacionado uma resposta puramente espinhal, enquanto o teste da chapa quente envolve uma resposta sensorial supraespinhal integrada, observada através de respostas como lambida da pata e pulos (KUBO et al., 2009; LANGERMAN et al., 1995). Isto possivelmente indica que a resposta antinociceptiva do DFIS envolva a modulação de mecanismos tanto espinhais quanto cerebrais no processamento da dor.

Relativo aos modelos químicos agudos de nocicepção, o composto DFIS, também apresentou efeito antinociceptivo nos testes de contorções induzidas por ácido acético, e testes de lambida da pata induzida por formalina, glutamato e capsaicina. Estes testes, usados para triagem de drogas com potencial antinociceptivo, apresentam especificidade e mecanismos de ação primários diferentes, embora possam atuar em vias comuns. Enquanto o teste do ácido acético apresenta-se com um teste com baixa especificidade, sendo relacionado a diversos resultados falso-positivos, o teste da formalina é considerado um dos mais preditivos de compostos com potencial antinociceptivo (LE BARS et al., 2001). Cabe salientar também que o teste da formalina apresenta duas fases, sendo uma neurogênica e outra relacionada a um período de sensibilização relacionada a participação de mediadores inflamatórios (MCNAMARA et al., 2007); observou-se primeiramente neste teste a influência do DFIS em uma situação de nocicepção inflamatória, a qual foi melhor abordada nos **manuscritos 1** e **2**. O DFIS também diminuiu as lambidas da pata induzidas por glutamato, sendo este teste relacionado a ativação de receptores glutamatérgicos (os quais podem ser ionotrópicos ou metabotrópicos), assim como canais iônicos sensíveis a ácido (ASICs) e receptores de potencial transitório do tipo vaniloide (TRPV1) (BLEAKMAN et al., 2006; MEOTTI et al., 2010). Além disso, o DFIS diminui a lambida da pata induzida por capsaicina, um teste mais especificamente relacionada a ativação dos receptores TRPV1, receptores presentes principalmente em fibras do tipo C e em menor quantidade em fibras do tipo Aδ, relacionados a transmissão de impulsos nociceptivos relacionados

a diferentes estímulos químicos (capsaicina e ácidos) e físicos (calor) (JULIUS e BASBAUM, 2001; TOMINAGA et al., 1998). Os receptores TRPV1 também estão relacionados a liberação de glutamato em fibras aferentes primárias e podem também ser sensibilizados por estímulos inflamatórios (JIN et al., 2009; JULIUS e BASBAUM, 2001). A via glutamatérgica em específico parece estar envolvida no efeito de alguns dos compostos orgânicos de selênio, como o $(PhSe)_2$ e o $(MeOPhSe)_2$, além disso, um trabalho recente também demonstrara que o $(PhSe)_2$ diminui a sensibilidade a dor resultante da administração neonatal de glutamato monossódico (PINTO et al., 2008; ROSA et al., 2015; SAVEGNAGO et al., 2007b). Em conjunto, estes resultados apontaram para o efeito antinociceptivo do DFIS, uma vez que também foram descartadas possíveis alterações na atividade locomotora através do teste do Campo Aberto, o que poderia levar a efeitos falso-positivos em testes de nocicepção (LE BARS et al., 2001).

No **artigo 2** foram realizados ensaios relativos a pesquisa do potencial antioxidante do DFIS *in vitro* e *in vivo*, uma vez que o estresse oxidativo apresenta um papel importante em vias de sinalização inflamatória e em doenças crônicas (KHANSARI et al., 2009; SCHIEBER e CHANDEL, 2014; VALKO et al., 2007). Como primeiros resultados, observou-se que o DFIS apresentou atividade antioxidante frente aos modelos de peroxidação lipídica induzida por Fe^{2+}/EDTA e carbonilação de proteínas induzida por nitroprussiato de sódio (NPS) em homogeneizados de cérebro, com concentrações inibitórias 50% (CI_{50}) relativamente baixas, de 1,35 µM e 0,74 µM, respectivamente. Um estudo de Prigol et al. (2009) demonstrou que dentre os disselenetos de diarila, os que apresentaram melhor efeito antioxidante foram o $(F_3CPhSe)_2$ e o disseleneto de *p*-cloro-difenila $(ClPhSe)_2$, com CI_{50} de 94 µM e 85 µM, respectivamente, para a carbonilação de proteínas induzida por NPS, bem maiores do que as observadas pelo DFIS. Também foram investigados possíveis mecanismos pelos quais o composto poderia estar agindo como antioxidante, nos quais observou-se que o DFIS apresenta atividade mimética às enzimas GST e DHAR, importantes enzimas fisiológicas de controle de estresse oxidativo, assim como atividade sequestrante de radicais de DPPH, ABTS e de NOx, os quais foram usados como exemplos de radicais livres (BITENCOURT et al., 2013; LUCHESE e NOGUEIRA, 2010b). Para reforçar o potencial do DFIS como uma molécula antioxidante, este foi avaliado frente a um modelo de dano oxidativo *in vivo*, a administração intracerebroventricular (i.c.v.) de NPS, no qual observou-se que o

composto reverteu alterações como aumento dos níveis de peroxidação lipídica e diminuição dos níveis de tiol não-proteico. Estes resultados corroboram com os experimentos *in vitro* de que o DFIS tem o potencial antioxidante em situações de dano oxidativo. O modelo utilizado, de dano oxidativo induzido por administração i.c.v. de NPS foi escolhido devido a capacidade desta molécula de liberar NO, cianeto e ferro, causando aumento das EROs e ERNs e dano a macromoléculas no SNC (HOTTINGER et al., 2014; PRIGOL et al., 2009). Além disso, um resultado intrigante é que os animais que receberam tanto administração i.c.v. de NPS quanto o DFIS pela via oral, apresentaram maior atividade da enzima GPx. Sabe-se que a GPx é uma selenoenzima responsável pela redução de diversos peróxidos às custas principalmente de glutationa, além de muitas vezes sua atividade ser usada como um marcador de biodisponibilidade do Se na dieta (PREEDY, 2015). Entretanto, devido ao tempo de exposição ao DFIS e ao NPS ser muito limitado, este efeito parece estar relacionado mais a uma ativação enzimática, do que a um aumento da expressão da enzima. Interessante também é que este efeito foi apenas observado nos animais que receberam o composto associado a uma situação de dano oxidativo. Embora não se possa afirmar com certeza que a administração oral de DFIS possa alcançar concentrações suficientes no cérebro, pode-se supor que o composto poderia estar detoxificando os danos causados pelo NPS por mecanismos similares aos observados *in vitro*. O DFIS poderia estar atuando no SNC, agindo como um agente mimético às enzimas GST e DHAR, assim como capturando radicais livres decorrentes da administração do NPS, incluindo o próprio NO.

Como anteriormente mencionado, nos **artigos 1** e **2**, foram observados efeitos positivos em relação ao DFIS, como efeito antioxidante observado em menores concentrações e uma prolongação no tempo seu efeito antinociceptivo, comparado a outros compostos orgânicos de selênio. Estas diferenças no efeito do DFIS, podem ser devido as diferenças estruturais do composto, como comentado na introdução, tanto compostos da classe dos disselenetos quanto compostos da classe dos selenazois apresentam atividade antioxidante e anti-inflamatória, sendo assim, um composto com ambos os grupamentos poderia apresentar melhores efeitos antioxidantes (NAM et al., 2008; NOGUEIRA et al., 2003; PARK et al., 2003; PRIGOL et al., 2009). Além disso, não podemos descartar que as diferenças estruturais do DFIS poderiam conferir efeitos tanto farmacodinâmicos quanto farmacocinéticos diferentes de outros compostos da mesma classe.

Tanto no **artigo 1** quanto no **artigo 2**, foram avaliados potenciais aspectos toxicológicos do composto DFIS. Enquanto no **artigo 1** foram abordados aspectos toxicológicos relacionados a administração oral do DFIS, no **artigo 2** foi investigado o efeito do composto sobre enzimas sulfidrílicas *in vitro*, também um importante parâmetro toxicológico a ser investigado em compostos orgânicos de selênio (BORGES et al., 2005; BRUNING et al., 2009a; CHAGAS et al., 2013; NOGUEIRA et al., 2004b). Quando foram avaliadas possíveis alterações em parâmetros toxicológicos como o peso corporal, o consumo de água e a comida, níveis de ureia e creatinina e atividade da alanina aminotransferase (ALT) e aspartato aminotransferase (AST) no plasma dos animais que receberam uma administração oral de DFIS: o único parâmetro alterado foi o consumo de água. Constatou-se que os animais que receberam DFIS na dose de 50 mg/kg, o qual pode ser explicado devido ao efeito anorexígeno que alguns compostos orgânicos de selênio possuem (BORTOLATTO et al., 2015; MEOTTI et al., 2008; SAVEGNAGO et al., 2009). No **artigo 2**, no qual foram realizados os experimentos *in vitro* relacionados a potencial toxicidade do DFIS, observou-se o efeito inibitório do composto sobre a atividade das enzimas sulfidrílicas δ-aminolevulinato-desidratase (δ-ALA-D) e Na^+,K^+-ATPase, bem como sobre a captação de [^{3}H]glutamato. Essas enzimas, assim como os transportadores responsáveis pela captação de glutamato, apresentam resíduos de cisteína, os quais além de serem importantes para sua completa funcionalidade, são extremamente sensíveis a oxidação. Sabe-se que os compostos orgânicos de selênio apresentam como característica química a capacidade de oxidar grupamentos sulfidrila, portanto a influência destes compostos sobre a atividade dessas enzimas e dos transportadores de glutamato é um ponto importante a ser investigado *in vitro* (NOGUEIRA e ROCHA, 2011; NOGUEIRA et al., 2004b). Entretanto, cabe salientar que embora o DFIS tenha efeito inibitório frente a estes parâmetros *in vitro*, estes efeitos foram observados em concentrações maiores do que as concentrações nas quais o mesmo apresentou atividade antioxidante.

Como anteriormente mencionado, nos **manuscritos 1** e **2**, pesquisou-se o possível efeito antinociceptivo do DFIS em modelos de nocicepção de cunho inflamatório. No **manuscrito 1** o composto foi avaliado frente ao modelo de nocicepção inflamatória induzida pela administração intraplantar de CFA. Por outro lado, no **manuscrito 2**, investigou-se o efeito da administração do DFIS nos animais que foram submetidos ao modelo de AIC.

Como primeiro resultado do **manuscrito 1**, observou-se que o DFIS reverteu a alodínia mecânica induzida pela administração do CFA na dose de 1 mg/kg mas não na dose de 0,1 mg/kg; neste trabalho também foram investigados parâmetros inflamatórios como atividade da MPO, níveis de NOx e 3-NT tanto no tecido da pata quanto na medula espinhal. Entretanto, quando avaliado frente as alterações teciduais induzidas pelo TCA, o DFIS não reverteu os parâmetros analisados no tecido da pata que estavam aumentados pela administração de CFA, mas reverteu o aumento dos níveis de NOx na medula espinhal. Relativo aos outros parâmetros analisados na medula espinhal, não foi observado aumento dos níveis de 3-NT na medula espinhal após a administração do CFA, assim como a atividade da MPO apresentou-se abaixo dos limites detectáveis em todos os grupos avaliados. Sabe-se que os níveis de NOx aumentados refletem indiretamente uma produção exacerbada de óxido nítrico, o que poderia estar relacionado ao efeito do DFIS sobre a produção ou manutenção nos níveis elevados de óxido nítrico na medula espinhal em resposta a um insulto inflamatório. Estudos já demonstraram que a administração de CFA, além de induzir inflamação local, também está relacionada a processos de sensibilização de vias nociceptivas tanto locais quanto centrais (RAGHAVENDRA et al., 2004). Além disso, a liberação de mediadores inflamatórios e nociceptivos por neurônios aferentes primários pode levar a ativação de células gliais, as quais podem auxiliar no processo de tornar células neuronais mais excitáveis e favoráveis a transmissão dos estímulos nociceptivos a outros locais do SNC (AOUAD et al., 2014; JI e WOOLF, 2001).

Devido ao papel do óxido nítrico em vias inflamatórias e como uma espécie reativa, resolveu-se investigar o envolvimento da via formadora desta molécula no efeito do DFIS. Assim, no **manuscrito 1**, observou-se que quando os animais tratados com DFIS recebiam um pré-tratamento com L-arginina (precursor do óxido nítrico), o efeito anti-alodínico do DFIS frente ao CFA era bloqueado. Sabe-se que a L-arginina leva a um aumento na produção de óxido nítrico devido a sua ação sobre a enzima formadora NOS, e que provavelmente a maior produção de óxido nítrico no modelo do CFA seja pela isoforma induzível iNOS (YONEHARA et al., 1997). O DFIS poderia estar agindo por alguns mecanismos, entre eles: a diminuição da atividade ou expressão da enzima iNOS, aumentados pela administração de CFA, assim como o DFIS poderia estar sequestrando os níveis elevados de óxido nítrico e espécies relacionadas, efeito que foi analisado no **artigo 2**. Por isso, um aumento

ainda maior da produção de óxido nítrico, devido ao processo inflamatório e a administração de L-arginina, poderia abolir os efeitos do DFIS.

O óxido nítrico, além de ser um neurotransmissor, apresenta-se como um importante modulador da atividade neuronal, podendo alterar a liberação e captação de neurotransmissores e vias de sinalização (CALABRESE et al., 2007). Por exemplo, a sinalização glutamatérgica é em grande parte influenciada pelo óxido nítrico, assim como diversos mediadores inflamatórios, os quais podem modificar a atividade dos transportadores de glutamato, geralmente diminuindo sua captação e podendo levar a uma maior excitabilidade neuronal (YASTER et al., 2011). Em um estudo de Raju e colaboradores (2015), fora reportado que a captação de [^{3}H]glutamato, especialmente via transportador de glutamato 1 (GLT-1), pode ser significativamente reduzida devido a S-nitrosilação dos resíduos de cisteína, e até mesmo a translocação dos receptores para a superfície da membrana pode ser modificada devido a este processo. Corroborando com o acima, no **manuscrito 1** foi observado uma diminuição na captação de [^{3}H]glutamato induzida pela administração de CFA, efeito o qual foi revertido pela administração do DFIS; o que poderia ser explicado devido ao seu papel em modular os níveis de óxido nítrico no SNC. No **artigo 1**, o DFIS diminuiu a nocicepção induzida pela administração intraplantar de glutamato, o que poderia estar relacionado a um envolvimento maior da sinalização glutamatérgica, e não apenas a um papel indireto no efeito do DFIS. Além disso, alguns compostos orgânicos de selênio apresentam efeito antinociceptivo e neuroprotetor em situações de aumento da excitabilidade glutamatérgica (DALLA CORTE et al., 2012; PORCIUNCULA et al., 2001; ROSA et al., 2015). Neste trabalho também foi investigada a atividade da Na^+, K^+-ATPase na medula, devido ao seu papel na excitabilidade neuronal, entretanto a atividade desta não foi alterada pela administração do CFA (VELDHUIS et al., 2003).

Ainda no **manuscrito 1**, também foram investigados parâmetros de estresse oxidativo na medula espinhal, como as atividades das enzimas GPx, GR, GST, os níveis de tiol não-proteico (NPSH) e de MDA. Embora a administração de CFA não tenha induzido alterações na atividade das enzimas antioxidantes que utilizam GSH como substrato, um resultado intrigante é que os animais que receberam tanto a administração de CFA quanto o DFIS apresentaram níveis aumentados de NPSH. Sabe-se que os NPSH, principalmente GSH, são utilizados na detoxificação de espécies reativas (DAVIS et al., 2001), e de alguma forma o composto poderia estar

auxiliando no aumento da produção de NPSH, particularmente em uma situação de dano oxidativo devido ao processo inflamatório exacerbado. Observou-se também um aumento nos níveis de MDA na medula espinhal dos animais que receberam administração intraplantar de CFA, e esta alteração foi revertido pelo DFIS. Como já fora mencionado anteriormente nesta tese, o processo inflamatório está relacionado a um aumento da produção tanto de EROs quanto ERNs devido a atividade enzimática das células imunes, através de enzimas como iNOS e NAPHox (CONNER e GRISHAM, 1996; KHANSARI et al., 2009). Outro fator que poderia estar contribuindo para o dano oxidativo seria o aumento da excitabilidade neuronal representada pela diminuição da captação de [^{3}H]glutamato, uma vez que se sabe que a excitotoxicidade também pode levar a um aumento da produção de espécies reativas (BONDY e LEBEL, 1993; NGUYEN et al., 2011). Esta produção aumentada de espécies reativas pode levar ao dano oxidativo de macromoléculas, sendo um dos principais a peroxidação lipídica, o qual apresenta como produto final o MDA (AYALA et al., 2014). O DFIS poderia proteger do dano oxidativo devido a uma série de mecanismos: uma possível interação com a via de produção do óxido nítrico, inclusive porque esta representa uma das mais importantes ERNs, assim como poderia estar auxiliando na detoxificação de outras espécies reativas que poderiam estar sendo formadas, mecanismos os quais foram abordados no **artigo 2**.

Tendo sido observado o efeito protetor do DFIS em um modelo de nocicepção inflamatória no **manuscrito 1**, optou-se por investigar-se o efeito do composto sobre um modelo de dor crônica de cunho inflamatório no **manuscrito 2**, tal como o modelo de AIC em camundongos (BRAND et al., 2007). Este modelo consiste de duas injeções de colágeno-tipo II (uma no dia 0 e outra no dia 21), o qual é variável dependendo da linhagem dos camundongos (e.g. colágeno de frango em camundongos C57BL/6 e colágeno de boi em camundongos DBA), para a indução de uma resposta autoimune evidenciada por nocicepção e inflamação nas articulações, semelhante aos sintomas relacionados a artrite reumatoide (BRAND et al., 2007; MCDOUGALL, 2006; SANDKUHLER, 2009). No estudo realizado neste manuscrito, observou-se tanto o desenvolvimento de alodínia mecânica quanto de hiperalgesia térmica, avaliados através do teste dos filamentos de Von Frey e pelo teste da Chapa Quente, respectivamente, após 60 dias da primeira administração de colágeno na cauda dos animais. Essas alterações nos parâmetros comportamentais, semelhante a alodínia mecânica induzida pelo CFA no **manuscrito 1**, também foram

revertidas pelo tratamento com o DFIS. Episódios de dor são frequentemente relatados por pacientes portadores de artrite reumatoide, e mesmo assim há uma baixa correlação entre a magnitude da dor e a dimensão do processo inflamatório local, sugerindo que a inflamação periférica contribui para a sensibilização a dor (BAS, D. B. et al., 2012; NIETO et al., 2016). Sabe-se que o aumento de citocinas pró-inflamatórias pode induzir alterações na excitabilidade de neurônios sensoriais nociceptivos, levando a alterações em correntes de íons e sistemas de segundos mensageiros destes neurônios, culminando no desenvolvimento da alodínia mecânica e da hiperalgesia térmica (SCHAIBLE, 2014; SCHAIBLE e GRUBB, 1993).

Algumas diferenças foram observadas nos resultados dos dois manuscritos, lembrando que o protocolo de administração do **manuscrito 2** envolveu um tratamento durante cinco dias, embora o melhor efeito tenha sido na dose de 1 mg/kg, também observou-se um efeito da menor dose de 0,1 mg/kg, o qual não havia sido observado no **manuscrito 1**. Relacionado as análises teciduais do protocolo crônico, outro fato que chamou atenção foi o fato de que DFIS protegeu do aumento da atividade da MPO na pata induzido pelo modelo de AIC, algo que não havia sido observado no protocolo agudo quando a inflamação foi induzida por CFA. Isto poderia indicar que em uma exposição maior o DFIS poderia levar a uma melhor administração do composto pelo organismo, sendo seus efeitos protetores observados de uma forma sistêmica, e não apenas retida ao SNC. Embora não tenham sido vistas alterações nos níveis de NOx na pata, neste protocolo o composto também protegeu do aumento nos níveis de NOx na medula espinhal induzidos pela AIC, corroborando com os resultados observados no protocolo agudo. Alguns estudos reportam níveis aumentados de óxido nítrico ou NOx em diversos tecidos, tanto no modelo de AIC quanto no modelo do CFA, e estudos demonstram que inibidores da síntese de óxido nítrico, sozinhos ou em combinação com outros agentes anti-inflamatórios, possuem efeito benéficos em modelos de artrite (CUZZOCREA et al., 2002; IALENTI et al., 1993; SAKAGUCHI et al., 2004; TOZZATO et al., 2016). A diminuição dos níveis exacerbados de ERNs relacionados ao óxido nítrico, parece corroborar com os achados do **artigo 2** e do **manuscrito 1**, demonstrando novamente que o efeito antioxidante do DFIS pode estar relacionado a seu efeito antinociceptivo, devido ao papel que o óxido nítrico tem tanto no processo inflamatório quanto na sinalização da dor.

No **manuscrito 2**, também foi investigado os efeitos do DFIS sobre algumas proteínas relacionadas a sinalização inflamatória, como os níveis do fator NFκB e da enzima COX-II (LAWRENCE, 2009). Sabe que o NFκB é um fator de transcrição com atividade induzível, presente na maioria das células, sendo sua atividade essencial para a sobrevivência das células, resposta inflamatória e imunidade inata (SMALE, 2011). Sua ativação é controlada principalmente a nível pós-transcricional através da formação de um complexo com a subunidade inibitória IκB no citoplasma, a qual quando fosforilada se desliga das subunidades p65 e p50 do NFκB e permite sua translocação para o núcleo onde estas promovem a transcrição de genes específicos (LAWRENCE, 2009; OECKINGHAUS et al., 2011). Observou-se que os animais do modelo de AIC apresentaram níveis aumentados de NFκB, mais especificamente da subunidade p65, na medula espinhal, o que provavelmente estaria relacionado a neuroinflamação resultado do processo inflamatório sistêmico. O tratamento com DFIS, além de diminuir os níveis de NFκB induzidos pelo modelo de AIC, também apresentou uma diminuição *per se* dos níveis deste fator. Além disso, o composto também diminuiu os níveis *per se* da enzima COX-II, corroborando para um potencial anti-inflamatório resultado de sua administração por um tempo mais prolongado. Na maior parte dos relatos da literatura, os níveis desses dois parâmetros estão correlacionados, ou seja, um aumento dos níveis de NFκB leva a um aumento da expressão da enzima COX-II (CROFFORD et al., 1997; KALTSCHMIDT et al., 2002; KE et al., 2007); entretanto, quando avaliou-se os níveis espinhais destas proteínas nos animais submetidos a AIC, esta relação não foi observada, assim apenas os níveis de NFκB apresentaram-se elevados. Alguns autores recomendam que conhecer a atividade dessas proteínas é tão importante quantos os seus níveis, uma vez que em algumas situações os níveis de uma proteína podem estar alterados, mas sua atividade não, assim como o contrário também pode ocorrer (CROFFORD et al., 1997; FITZPATRICK et al., 2010). Entretanto, nos protocolos realizados, apenas avaliamos os níveis das duas proteínas, o que pelo menos nos auxilia no entendimento dos mecanismos pelos quais o composto DFIS poderia estar agindo.

O uso de compostos antioxidantes para o tratamento de doenças inflamatórias como a artrite tem sido alvo de interesse e discussão, principalmente devido as evidências que demonstram tanto o envolvimento do estresse oxidativo no processo inflamatório quanto a relação entre o consumo de antioxidantes na dieta e

a probabilidade de desenvolvimento de artrite inflamatória (PATTISON e WINYARD, 2008). Estudos também demonstram que diversas das moléculas antioxidantes modulam seus efeitos anti-inflamatórios e antinociceptivos tanto pela diminuição dos níveis de espécies reativas quanto por mecanismos que envolvem a regulação da atividade ou níveis do NFκB, ou ainda por evitar que certas vias sejam ativadas ou inibidas por agentes oxidantes (BRUNING et al., 2015; GERONIKAKI e GAVALAS, 2006; PATTISON e WINYARD, 2008; VALKO et al., 2007). O presente estudo averiguou o efeito do DFIS como um potencial efeito antinociceptivo e antioxidante nos **artigos 1** e **2**, e o seu potencial uso em doenças relacionadas a dor inflamatórias nos **manuscritos 1** e **2.** Os parâmetros avaliados indicam que o DFIS deva suas propriedades antinociceptivas e anti-inflamatórias, pelo menos em parte, a sua interação com o sistema antioxidante, seja diminuindo os níveis de espécies reativas ou diminuindo efeitos secundários ao estresse oxidativo: como os danos teciduais, o aumento da excitabilidade neuronal e exacerbação de vias inflamatórias como a via do NFkB.

A Figura 8 apresenta um resumo dos efeitos observados pela administração do DFIS abordados nesta tese e os possíveis mecanismos envolvidos nestes efeitos.

Figura 8. Resumo dos efeitos observados pela administração do DFIS abordados nesta tese.

5. CONCLUSÃO

Os resultados apresentados nesta tese indicam que o composto DFIS:

(i) Apresentou efeito antinociceptivo em modelos de nocicepção térmica e química sem toxicidade aparente nas doses testadas;

(ii) Demonstrou efeito antioxidante em protocolos *in vitro* e *in vivo* e apenas inibiu enzimas sulfidrílicas em concentrações maiores do que as relacionadas ao seu efeito antioxidante;

(iii) Reverteu a alodínia mecânica induzida por CFA, sendo este efeito bloqueado pela administração de L-arginina, indicando uma participação da via do óxido nítrico e do papel antioxidante no efeito do DFIS;

(iv) Possuiu efeito protetor frente ao modelo de AIC, revertendo os parâmetros comportamentais alodínia mecânica e hiperalgesia térmica, e seu efeito parece estar relacionado a modulação da atividade da MPO, dos níveis de NOx e NFκB.

Sabe-se que doenças relacionadas a dor e a inflamação possuem diversos mecanismos patofisiológicos, sendo o estresse oxidativo um destes mecanismos. Por isto moléculas antioxidantes poderiam constituir uma nova ferramenta de auxílio ao tratamento. Considerando os efeitos do DFIS demonstrado no presente estudo, esse composto orgânico de selênio poderia ser uma futura alternativa terapêutica para o tratamento destas patologias.

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ANEXO A – Carta de Aprovação da Comissão de Ética no Uso de Animais-UFSM:

UNIVERSIDADE FEDERAL DE SANTA MARIA
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS-UFSM

CARTA DE APROVAÇÃO

A Comissão de Ética no Uso de Animais-UFSM, analisou o protocolo de pesquisa:

Título do Projeto: "Avaliação das propriedades antinociceptivas e anti-Inflamatórias do Disseleneto de Bis(Fenilimidazoselenazolila) em modelos crônicos."

Número do Parecer: 066/2014

Pesquisador Responsável: Prof.ª Dr.ª Cristina Wayne Nogueira

Este projeto foi **APROVADO** em seus aspectos éticos e metodológicos. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente a este Comitê.

OBS: Anualmente deve-se enviar à CEUA relatório parcial ou final deste projeto.

Os membros da CEUA-UFSM não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

DATA DE APROVAÇÃO: 11/09/2014.

Santa Maria, 11 de setembro de 2014.

Vania Lucia Loro

Prof.ª Dr.ª Vania Lucia Loro
Vice-Coordenadora da Comissão de Ética no Uso de Animais- UFSM

ANEXO B - Autorização para reprodução do artigo científico: "Bis(Phenylimidazoselenazolyl) Diselenide: A Compound with Antinociceptive Properties in Mice" publicado na *Behavioural Pharmacology*, v. 24, 37-44, 2013

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Title: Bis(phenylimidazoselenazolyl) diselenide: a compound with antinociceptive properties in mice

Author: Pietro Chagas, Cristiani Bortolatto, Ethel Wilhelm, et al

Publication: Behavioural Pharmacology

Publisher: Wolters Kluwer Health, Inc.

Date: Jan 1, 2013

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