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EFEITO ANTINOCICEPTIVO DA N-ACETILCISTEINA E DA VITAMINA E EM MODELOS DE NOCICEPÇÃO EM ROEDORES

TESE DE DOUTORADO

MATEUS FORTES ROSSATO

Santa Maria, RS, Brasil, 2013

EFEITO ANTINOCICEPTIVO DA N-ACETILCISTEINA E DA VITAMINA E EM MODELOS DE NOCICEPÇÃO EM ROEDORES

POR

MATEUS FORTES ROSSATO

Tese apresentada no curso de Doutorado do Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de **Doutor em Ciências Biológicas: Bioquímica Toxicológica**.

Orientador: Prof. Dr. Juliano Ferreira

Santa Maria, RS, Brasil, 2014

Universidade Federal de Santa Maria

Centro de Ciências Naturais e Exatas

Programa de Pós-Graduação em Ciências Biológicas:

Bioquímica Toxicológica

A comissão examinadora, abaixo assinada, aprova a tese de doutorado intitulada **EFEITO ANTINOCICEPTIVO DA N-ACETILCISTEINA E DA VITAMINA E EM MODELOS DE NOCICEPÇÃO EM ROEDORES,** elaborada por Mateus Fortes Rossato como requisito parcial para obtenção do grau de Doutor em Bioquímica Toxicológica.

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Santa Maria, RS, Brasil, 2014

Definitivo

Definitivo, como tudo o que é simples.

Nossa dor não advém das coisas vividas,

mas das coisas que foram sonhadas e não se cumpriram

...

A dor é inevitável

O sofrimento é opcional

(Carlos Drummond de Andrade)

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O decurso de nossas vidas é marcado pelas obras que realizamos e pelas pessoas que tocamos, por nossas lembranças. Em todos esses pontos, nunca estamos sozinhos, mesmo quando assim pensamos. Logo, esse trabalho não seria diferente; esta obra é de minha mente e punho, mas tocado e enriquecido por muitos. É inviável pensar em agradecimentos sem iniciar por minha família como um todo, em especial meus pais (Seu João e Dona Rose, os veios) e meu irmão (Giu, o Guri). Desde sempre apoiaram minhas opiniões e sonhos de forma incondicional. Da mesma forma, alguns amigos acompanharam toda a trajetória de construção dessa tese, com admiração e incentivo: os meus queridos Carecas, os Patetas da Superliga de Vôlei e os antigos Ney's (sim, meus grupos de amigos têm nomes). De uma forma muito orgulhosa, posso incluir no grupo de amigos a equipe LabNeuro, na qual iniciei minha iniciação científica e terminarei meu doutorado, tudo isso sob orientação do professor Juliano, a quem tenho muito a agradecer, por acreditar no meu potencial e me incentivar a sempre crescer, me ensinar a fazer ciência. Muito mais que companheiros de trabalhos, uma ligação de amizade se fez dentro do grupo, nos tornando ainda mais coesos, eficazes e potentes. Apesar disso, dentre todos destaco alguns, como Carin (minha Lua), Flávia (A mãezona), Gabriela ("Quem sou eu?") e Jonatas (o outro Y). Por último, mas não menos importante, aos professores que nos auxiliaram durante o processo de realocação do núcleo Dor/SM do LabNeuro: Maribel Rubin, Mauro Oliveira e Marcus Vinicius Gomez. A todos que lerão essa tese e aos professores que a aprovaram, muito obrigado!

RESUMO

Tese de Doutorado

Programa de Pós-graduação em Ciências Biológicas: Bioquímica Toxicológica

Universidade Federal de Santa Maria, RS, Brasil.

EFEITO ANTINOCICEPTIVO DA N-ACETILCISTEINA E DA VITAMINA E EM MODELOS DE NOCICEPÇÃO EM ROEDORES

Recentemente, o estresse oxidativo foi indicado como possível modulador da neurotransmissão dolorosa, havendo também a constatação de que o óxido nítrico (NO) pode ativar, *in vitro,* o receptor de potencial transitório vaniloide 1 (TRPV1). Como os compostos tiólicos não proteicos (SH) são antioxidantes endógenos importantes, decidimos investigar a relação entre os tióis endógenos, exógenos e a possível ativação do receptor TRPV1 mediada pelo NO, na medula espinhal de camundongos. Observamos que a administração sistêmica (i.p.), intratecal (i.t.), mas não intraplantar (i.pl.) de N-acetilcisteína (NAC) reduz a nocicepção, ocasionando a diminuição dos níveis espinhais de tióis não proteicos (SH), e o aumento de radicais livres ao ácido tiobarbitúrico (TBARS) e 3-nitrotirosina (3-NT) induzido por capsaicina (CAP) intraplantar. Similarmente, a administração i.p. ou i.t. de NAC reverte tanto a nocicepção (alodínia mecânica) quanto à diminuição nos níveis espinhais de SH induzida pela administração intraplantar de adjuvante completo de Freund (CFA). Comprovando esses dados, observamos que a butionina-sulfoxamina (BSO), inibidor da síntese do principal composto tiólico endógeno (glutationa), induziu a diminuição nos níveis de SH espinhal. Além disso, o BSO também induziu a alodínia química e mecânica, e a hiperalgesia térmica e mecânica. Para investigar o papel do NO nessas alterações, induzimos a nocicepção (hiperalgesia térmica) pela administração intraplantar de CAP ou intratecal de L-arginina (ARG), substrato da enzima óxido nítrico sintase (NOS). Em ambos os casos, o pré-tratamento i.t. com NAC ou L-NAME (inibidor da NOS) inibiu essa nocicepção, bem como a diminuição nos níveis de SH e a elevação nos níveis dos metabólitos estáveis do NO nitrito/nitrato (NOx). Essas respostas também foram prevenidas pela inibição farmacológica (antagonista SB366791) e pela desfuncionalização (administração intratecal prévia de capsaicina) ou ablação gênica (administração repetida de oligonucleotídeo antissentido) do receptor TRPV1. Dessa forma, podemos concluir que o conteúdo tiólico não proteico participa da transmissão dolorosa neutralizando o NO formado e impedindo a ativação do TRPV1 espinhal. Sendo assim, antioxidantes como a NAC podem exercer seu efeito antinociceptivo por impedir esse processo de ativação.

Palavras-chave: Nocicepção, medula espinhal, *óxido nítrico, N-acetilcisteína, capsaicina*.

ABSTRACT

PhD Thesis

Graduate Course in Biological Sciences: Toxicological Biochemistry

Federal University of Santa Maria, RS, Brazil

Effect of N-acetylcysteine and vitamin E in animal models of nociception

Recently, oxidative stress was indicated as modulator of nociceptive transmission at spinal cord, and that nitric oxide (NO) may activate the TRPV1 in vitro. As no protein thiol compounds (SH) are the main endogenous antioxidants, we decided to investigate the relation between endogenous and exogenous SH, as well as the NO-mediated TRPV1 activation at spinal cord in mice. We observed that the systemic (i.p.), intrathecal (i.t.), but not local (I.pl.) N-acetylcysteine (NAC) administration reduced the nociception, the decrease in spinal SH, raise of thiobarbituric reactive species (TBARS) and 3-nitrotyrosine (3-NT) levels induced by intraplantar capsaicin (CAP). Similarly, i.t. or i.p. NAC administration reduced the nociception (mechanical allodynia) and decrease in spinal SH induced by complete Freund adjuvant (CFA)-induced chronic inflammation. Reinforcing these results, we observed that buthionine-sulfoxamine (BSO), an inhibitor of glutathione synthesis, the main endogenous SH compound, induced a decrease in spinal SH levels, chemical and mechanical allodynia, thermal and mechanical hyperalgesia. To investigate the participation of NO in these processes, we induced spinal nociception (thermal hyperalgesia) by i.t. L-arginine (ARG – substrate for endogenous NO synthesis) administration and intraplantar CAP administration. In both cases, the i.t. pre-treatment with NAC or L-NAME (NO synthesis inhibitor) prevented this nociception, as well as the decrease in spinal SH and the raise in nitrite/nitrate (NOx) levels, a stable metabolites of NO. These changes were also prevented by the pharmacological blockade of TRPV1 with the antagonist SB366791, the spinal defunctionalization (induced by i.t. high dose of CAP) and genetic knockdown (induced by repeated oligonucleotide antisense i.t. administration). Thus, we may conclude that SH compounds participate in the spinal nociceptive transmission by neutralizing NO, preventing spinal TRPV1 activation. Therefore, antioxidants as NAC may present antinociceptive effect by this process.

Keywords: *Nociception, spinal cord, nitric oxide, N-acetylcysteine, capsaicin.*

LISTA DE ABREVIATURAS

12-HPETE.....Ácido 12-hidroperoxi-eicosatetraenóico

ANOVA.....Análise de variância

AS.....Oligonucleotídeo antisentido

ATP.....Trifosfato de adenosina

BSO.....Butionina-sulfoxamina

CAT.....Catalase

CFA.....Adjuvant completo de Freund

CGRP.....Peptídeo relacionado ao gene da calcitonina

DRG.....Gânglio da raiz dorsal

GPx.....Glutationa peroxidase

GOx.....Glutationa oxidase

i.p. .....Intraperitoneal

i.pl. .....Intraplantar

i.t. .....Intratecal

H_2O_2.....Peróxido de hidrogênio

L-NAME.....L-N^{ω}-nitroarginina metil ester

MM.....Sequencia incorreta dos aminoácidos do oligonucleotídeo antisentido

NADA.....N-araquidonoil dopamina

NAC.....N-acetilcisteina

NADPH.....Difosfato reduzido de nicotinamida e adenina

NMDA.....N-metil-D-aspartato

$NO^{\bullet}$.....Óxido nítrico

NOS.....Óxido nítrico sintase

NOx.....Relação nitrato / nitrito

NOX.....NADPH oxidase

NTG.....Nitroglicerina

O_2.....Oxigênio molecular

$O_2^{\bullet}$.....Radical superóxido

SIN1.....Cloiridrato de 3-Morfolinosidnonimina

SNC.....Sistema nervoso central

SOD.....Superóxido dismutase

PBS.....Solução salina tamponada com fosfato

SNP.....Sistema nervoso periférico

TRP.....Receptor de potencial transitório

TRPA.....Receptor de potencial transitório anquirina

TRPC.....Receptor de potencial transitório canônico

TRPM.....Receptor de potencial transitório melastatina

TRPML.....Receptor de potencial transitório mucolipina

TRPP.....Receptor de potencial transitório policistina

TRPN.....Receptor de potencial transitório não sensível a estímulo mecânico

TRPV.....Receptor de potencial transitório vanilóide

TRPV1.....Receptor de potencial transitório vanilóide subtipo 1

SUMÁRIO

APRESENTAÇÃO

Este trabalho será apresentado na forma de INTRODUÇÃO, contendo uma revisão bibliográfica abrangendo todos os tópicos abordados durante o trabalho. A metodologia utilizada e os resultados obtidos que compõem esta tese estão apresentados sob a forma de artigo científico publicado e manuscrito, os quais se encontram no item ARTIGOS e MANUSCRITO. As seções Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se no próprio artigo e manuscrito e representam a íntegra deste estudo. O item CONCLUSÕES é encontrado no final desta tese e apresenta interpretações e comentários gerais sobre os artigos científicos contido neste trabalho, e as REFERÊNCIAS BIBLIOGRÁFICAS referem-se somente às citações que aparecem no item INTRODUÇÃO e CONCLUSÃO desta tese.

1. INTRODUÇÃO

1.1. Dor

1.1.1. A dor e sua história

Desde o início da formação das primeiras comunidades nômades, fomos guiados pela busca de satisfação de nossas necessidades mais básicas, gerando o conflito entre o homem e a natureza. Desde esses tempos remotos até os dias de hoje, a sobrevivência humana depende da capacidade de se adaptar ao ambiente, de vencer os obstáculos impostos pela natureza e de saciar, de alguma forma, nossas necessidades. Da interface entre homem e natureza, surgem as diferentes sensações conhecidas por nosso corpo e nossa mente. Dentre todas as sensações, a dor se destaca como uma sensação marcadora de nossa luta pela sobrevivência e sucesso adaptativo. Isso se deve ao fato de que, ao contrário de outras sensações fisiológicas, a dor se refere não somente a uma estimulação física de nosso organismo, mas também emocional, sendo referida, muitas vezes, como lesão ou como sofrimento.

Devido à importância que ela assume em nossa vida e os reflexos que causa, a dor vem sendo estudada desde os tempos remotos, e ainda hoje se mostra de forma misteriosa. É fato que encontramos sinais da pesquisa por esse assunto desde as primeiras civilizações, passando pelos grandes filósofos de nossa era, até os dias de hoje, evidenciados através desta tese (Bonica, 1990). Os primeiros relatos textuais sugiram no Antigo Egito, onde o coração era apontado como o centro das sensações humanas, ponto chave que concentrava todos os estímulos internos e externos de nosso corpo. Para os

egípcios, a dor resultava de uma punição divina, devido ao descontentamento dos deuses frente às ações humanas. Dessa forma, essa punição poderia vir sob a forma de pestes e doenças, ou como um tormento interno e psíquico, mostrando a dualidade do que hoje conhecemos como dor. Devido a isso, o tratamento da dor consistia em uma combinação de ervas, amuletos e orações, apaziguando corpo e espírito (Bonica, 1990).

Já na Grécia Antiga, filósofos como Hipócrates postularam a teoria dos humores corporais, fluídos que banhariam e percorreriam nosso corpo (sangue, flegma, bile verde e nega), os quais seriam os responsáveis por nossa saúde e bem-estar. Do desequilíbrio desses fluídos gerar-se-iam as diferentes doenças, cada qual originada por uma diferente combinação de tais fluidos, e como sinal dessas enfermidades, a dor. Já Platão e Aristóteles refutavam essa ideia, definindo a dor como a sensação oposta ao prazer, como um reflexo físico do sofrimento da alma (Bonica, 1990). Apesar disso, as ideias de Hipócrates inspiraram diferentes pesquisadores pelo mundo, principalmente em Alexandria, um dos grandes berços de nossa ciência, onde as primeiras dissecações humanas foram relatadas. Anatomistas como Herófilo descobriram estruturas conectando a periferia do nosso organismo ao sistema nervoso central, colocando o cérebro, pela primeira vez na história, como o grande centro integrador de nossas sensações, dentre elas a dor (Rey, 1995).

Nos séculos seguintes, a expansão religiosa de Roma e, subsequentemente, do cristianismo causaram uma estagnação científica das pesquisas referentes ao corpo humano. A partir de então se voltou a propagar a dor como um ato de provação divina a ser encarado com fé e resistência (Ray, 1995). Essa visão perdurou até a Renascença, quando pesquisadores e

filósofos como Leonardo da Vinci retomaram e aprofundaram as ideias de Hipócrates e Herófilo. Nessa época, o sistema nervoso foi mais estudado, descrevendo-se os nervos como sistemas tubulares formadores da medula espinhal, responsáveis por encaminhar os estímulos periféricos até o cérebro, sendo que seu terceiro ventrículo seria o centro responsável por “sentir” a dor (Proccati e Maresca, 1984).

As ideias de Hipócrates e Herófilo evoluíram com Descartes e sua visão mecanicista, a qual postulou a formação dos nervos periféricos como estruturas cilíndricas formadas por pequenos túbulos, sendo cada um responsável por conduzir uma sensação distinta até o cérebro, refutando totalmente a ideia de o coração como o centro sensorial. Essas ideias permaneceram incólumes até o período entre os séculos XIX e XX, quando se iniciaram os primeiros experimentos visando à mensuração da dor em animais e humanos, com o intuito de descrever mais apropriadamente as diferentes formas de dor, bem como os diferentes tratamentos (farmacológicos ou não) que alteravam essa sensação (Rey, 1995).

1.1.2. Era da experimentação: primeiras teorias sobre dor

A partir de meados dos séculos XIX e XX, a era das experimentações teve inicio de forma mais sólida, com o advento de aparatos específicos e situações mais controladas. Dessa forma, vários pesquisadores de distintas áreas tentaram descrever de forma mais acurada as diferentes sensações reconhecidas por nosso organismo, bem como descrever suas vias neuroanatômicas. Neste novo panorama, novamente a dor apresenta um papel

intrigante, uma vez que a subjetividade de sua natureza dual (física e emocional/psicológica) permite interpretações múltiplas de sua complexidade (Rey, 1995).

Apesar de as primeiras teorias acerca da origem da dor remontarem a filósofos como Aristóteles, decorrendo por toda a história humana, a maioria delas girava em torno da questão emocional. Tais teorias, apesar de antigas, permaneceram em foco, classificando a dor como o estado oposto ao do prazer e bem-estar, refletindo um estado de integridade física e emocional. Essas teorias foram postuladas por Strong em 1895, definindo assim a dor como a sensação primordial do ser humano, designando então a teoria afetiva da dor (Onofre, 2009).

Além disso, muitos pesquisadores postularam diferentes tipos de teorias explicativas referente à dor, a maioria delas agregadas posteriormente no que hoje chamamos de “Teorias da Intensidade” e “da Especificidade”. Esses dois aspectos observados postulavam, muitas vezes de formas contraditórias, as origens e os mecanismos de sinalização da dor. A primeira teoria (Intensidade) regia que todo estímulo, independente de sua modalidade e origem, caso chegasse a uma intensidade suficientemente forte, poderia ativar as vias neuronais da dor e desencadear tal sensação. Enquanto isso, a segunda hipótese (Especificidade) postulava que a dor consistia de uma modalidade independente de sensação, tal qual o tato e a termocepção, com um sistema de percepção e interpretação independente e elaborado (Silva e Ribeiro Filho, 2011).

Essas teorias muitas vezes conflitantes impulsionaram as pesquisas de diferentes pesquisadores, como as realizadas pelo neurofisiologista Maximilian

Ruppert Franz von Frey, o qual utilizou um sistema de filamentos para a estimulação mecânica da pele, a partir de crina de cavalo, cada qual com espessura diferente. Dessa forma, von Frey conseguiu mapear a superfície da pele, descrevendo regiões com diferentes sensibilidades, caracterizando estimulações nocivas e inócuas. Na década de 1880, von Frey descreveu a pele como um mosaico sensorial, com regiões capazes de sentir diferentes estimulações, dependendo de sua inervação. Dentro deste mosaico, o reconhecimento de estímulos dolorosos ficaria a cargo de terminações nervosas livres com alto limiar de ativação (Onofre, 2009).

Posteriormente, outros pesquisadores como Titchener e Goldscheider ampliaram as primeiras descobertas de von Frey, descrevendo a existência de nódulos sensitivos presentes não somente na pele, mas também em mucosas. Esses nódulos seriam terminações nervosas livres dotadas da capacidade de reconhecer estímulos sensoriais térmicos e mecânicos de diferentes intensidades: os corpúsculos de Krause. Essas primeiras descobertas reforçavam as ideias de von Frey e seus demais colegas “especificistas”. Ainda assim, essas teorias ainda não respondiam a eventos como a somação temporal da estimulação mecânica / nociva, bem como a exacerbação da intensidade dolorosa frente à estimulação de diferentes tipos (Freeman e Okun, 2002).

Além disso, a necessidade de uma estimulação também refutava a ideia de que a dor pudesse ser uma emoção. A partir dessas observações, Goldscheider (1894) passou a postular a dependência da intensidade da estimulação, classificando-a em três níveis distintos: o prurido, sensação decorrente da estimulação mecânica branda; o tato, decorrente da estimulação

mecânica moderada; e a dor, decorrente da estimulação mecânica intensa. Ainda, todos esses estímulos seriam percebidos pelos mesmos receptores periféricos, porém conduzidos de forma seletiva por diferentes vias neuronais até o sistema nervoso central, onde seriam diferenciados e reconhecidos (Onofre, 2009).

Apesar disso, todas as teorias continuaram a ser pesquisadas e novas afirmações, por parte de psicólogos, fisiologistas e neurologistas, continuaram a embasar e refutar as teorias vigentes, fazendo com que o século XX começasse com três teorias paralelas: as Teorias da Especificidade, da Intensidade e a Afetiva. O panorama teórico permaneceu em debate, até meados do século XX, quando a dor passou a ser considerado um evento multifatorial, composto por estimulação sensorial e/ou por experiências emocionais desagradáveis. Nesse contexto, teorias convergentes e elaboradas surgiram, não mais com o objetivo de definir qual a real característica da dor, mas sim seus mecanismos e sua fisiologia.

Um dos primeiros e mais importantes passos nesse sentido foi dado no começo do século XX por Charles Sherrington e sua equipe de neurofisiologistas. Em seu trabalho intitulado "*The Integrative Action of the Nervous System*" (1906), descreveram a existência de terminações nervosas livres com diferentes graus de mielinização, localizadas em diferentes tecidos e profundidades do organismo. Sherrington utilizou técnicas eletrofisiológicas para determinar, inicialmente em animais e posteriormente em humanos, as características dessas terminações, desvendando sua especificidade quanto ao tipo de estímulo. A partir disso, o pesquisador postulou que determinadas terminações (fibras) seriam capazes de ser estimuladas caso a classe da

estimulação e sua intensidade fosse adequada, e que parte dessas fibras seriam responsáveis por detectar estímulos nocivos e de lesão tecidual. Estas fibras, em especial, foram denominadas “nociceptores” (Levine, 2007).

Posteriormente, o início da elucidação de eventos periféricos permitiu um aprofundamento no estudo da dor e o mapeamento de outras estruturas fundamentais, como a medula espinhal. Um exemplo disso foi a Teoria do Portão, elaborada por Wall e Melzack, em 1965, para tentar explicar o funcionamento da medula espinhal e o sistema de controle da dor. Segundo essa teoria, a estimulação mecânica cutânea seria transmitida por fibras mielinizadas e de grande diâmetro, com rápida velocidade de condução (A_α e A_β) até as lâminas mais profundas do corno dorsal da medula espinhal (lâmina IV e V), onde estimulariam subtipos específicos de interneurônios, liberando substâncias inibitórias. Enquanto isso, os estímulos nociceptivos seriam transmitidos por fibras pouco mielinizadas ou não mielinizadas de pequeno diâmetro (A_δ e C) e de baixa velocidade de condução. Essas fibras chegariam às lâminas mais superficiais do corno dorsal da medula espinhal (lâmina I e II), onde promoveriam a liberação de substâncias excitatórias (Mendell, 2014).

Graças a essa diferença entre as velocidades de condução, a estimulação mecânica inócua conseguiria promover a liberação de substâncias inibitórias antes da chegada dos estímulos nocivos à medula espinhal, aumentando o limiar de ativação. Dessa forma, a percepção da dor só aconteceria quando a estimulação nociva chegasse a um ponto intenso o suficiente para transpor a inibição, funcionando literalmente como um portão (Mendell, 2014).

1.1.3. Visão moderna da dor: a neuromatriz

A Teoria do Portão remodelou de forma geral a visão sobre a dor, tanto dentro da comunidade científica quanto médica. Ela trouxe à luz questões mais fisiológicas do que descritivas, colocando centros nervosos como a medula espinhal de forma mais ativa no processamento da dor, não apenas como pontos de transmissão e reconhecimento. A medula passou a ser um centro integrador e regulatório, enquanto o cérebro ficou a cargo de reconhecer e discriminar a sensação.

Todavia, o próprio Ronald Melzack, idealizador da Teoria do Portão, encontrou lacunas importantes em sua teoria, estudando pacientes com dor do membro fantasma ou com lesão parcial/total medular. Nestes casos, mesmo com a perda de um membro, ou da inervação referente a este membro, a sensibilidade dolorosa permanecia, bem como outras sensações (mecano e termossensação). Esse fato também era observado em casos de pacientes com dores crônicas e sem lesão aparente. Muitas vezes, tais casos eram tratados como alterações psiquiátricas, levando pacientes a tratamentos insatisfatórios e a sofrimento desnecessário (Melzak, 1999). Assim, Melzack acreditava que outros centros nervosos poderiam estar integrados aos circuitos referentes à dor e os ativando, independente de uma estimulação periférica. Reforçando essa ideia, alguns experimentos demonstraram que, em animais, a ablação de alguns centros nervosos responsáveis pela memória e emoção era capaz de atenuar ou extinguir a sensação dolorosa (Melzak, 1999; Lannetti GD, Mouraux, 2010).

A partir desse grupo de fatos e informações, Melzack teorizou que o sistema responsável pelo reconhecimento e compreensão da dor não seguiria um sistema cartesiano tão linear quanto o descrito anteriormente em sua teoria, mas seria composta por uma série de *loops* entre neurônios de diferentes regiões cerebrais, integrando diferentes sistemas como a dor, a memória e a emoção. Dessa forma, a dor poderia ser uma "sensação" complexa e multimodal fatorial, modulada não apenas pela nossa capacidade de reconhecer estímulos nocivos oriundos do ambiente, mas também modulada/ativada por questões referentes ao nosso estado emocional, por nossas lembranças e experiências de vida (Lannetti GD, Mouraux, 2010).

Assim, existiria uma "neuromatriz" responsável por coordenar e integrar todos esses fatores, e esta nos daria a percepção global da dor. A ativação cíclica dessa neuromatriz e o reforço exercido pelas memórias e emoções referentes à dor, somadas as configurações genéticas do indivíduo, criariam um padrão, ou uma "neuroassinatura", responsável por caracterizar a forma como esse indivíduo percebe e reage à dor (Melzak, 2001). Sendo assim, hoje, a Associação Internacional para o Estudo da Dor (IASP) define a dor como uma experiência sensorial e/ou emocional desagradável, associada ou não a uma lesão tecidual real ou potencial, ou descrita em termos de tal lesão (Loeser e Treede, 2008).

1.1.4. Dor: do nociceptor à medula espinhal

Apesar de sua visão moderna, na qual a dor é caracterizada como uma sensação multimodal, abrangendo diferentes sistemas neuronais e emocionais,

memórias, prazeres, experiências e outros aspectos humanos, pode-se fragmentar a dor em dois componentes: o emocional e o fisiológico. Desse modo, caracteriza-se a dor como a sensação física ou emocional provocada por uma estimulação desagradável ou lesiva ao organismo. A dor, em seu caráter fisiológico (nocicepção), possui o caráter protetivo ao sinalizar lesão tecidual e desencadear comportamentos de proteção ao sítio da lesão (dor nociceptiva) (Loeser e Treede, 2008).

Perifericamente, a capacidade de perceber estímulos nocivos ao nosso organismo está a cargo de uma série de terminações nervosas livres, formadas por fibras do tipo Aδ (pouco mielinizadas) e C (não mielinizadas), cujo corpo celular se encontra no gânglio da raiz dorsal (DRG), e são denominadas “nociceptores”. Estas terminações apresentam em sua estrutura uma gama de receptores capazes de reconhecer e de serem ativados por diferentes estímulos, como térmicos (frio e calor), químicos (agentes oxirredutores, alterações de pH) e mecânicos. Além disso, agentes internos, como os mediadores inflamatórios, as aminas e os peptídeos liberados por diferentes tipos celulares, também são capazes de ativar e modular a ativação dos nociceptores (Scholz e Woolf, 2002).

Uma vez que esse estímulo seja intenso o suficiente para ativar esses receptores, forma-se um potencial gerador que percorre a fibra nervosa, passa pelo DRG e chega até a primeira sinapse na medula espinhal. Neste sítio, as terminações nervosas formadoras dos nociceptores chegam principalmente nas lâminas I e II do lado ipsilateral do corno dorsal da medula espinhal. Neste ponto, o potencial gerador desencadeado pela estimulação nociva desencadeia a liberação de neurotransmissores (glutamato, substância P, CGRP, dentre

outros) pelo terminal pré-sináptico, transmitindo esse sinal até a terminação pós-sináptica (Reichling et al., 2013). Tal ponto da transmissão nociceptiva é muito importante, pois é nele onde ocorre a integração de todos os estímulos periféricos, formando um único estímulo que será enviado a centros supra-espinhais, como o tálamo, o sistema límbico, o hipocampo e o córtex frontal, onde serão devidamente reconhecidos e discriminados (Figura 1) (Hunt e Mantyh, 2001).

Apesar disso, em condições patológicas podem ocorrer alterações na capacidade de percepção de estímulos nocivos, fazendo com que estímulos inócuos sejam percebidos como nocivos (alodínia) ou desenvolvendo uma sensibilidade aumentada a estímulos dolorosos (hiperalgesia). Isso se deve a alteração da sensibilidade de ativação de receptores presentes nestes dois sítios (nociceptor e medula espinhal) por eventos transitórios (fosforilação/oxidação de receptores, alteração na liberação de neurotransmissores/neuromoduladores), ou de plasticidade (alteração no padrão de expressão de proteínas, forma e função de fibras). A todo esse processo de alteração de funcionalidade, denominamos “sensibilização periférica e central” (Loeser e Treeder, 2008).

Dentre os diferentes alvos já identificados que participam desses processos, tanto de detecção e transmissão da dor, quanto de alteração de função durante eventos patológicos encontra-se de maneira relevante alguns dos membros da superfamília de receptores de potencial transitório (TRP). Estes se encontram presentes tanto na periferia do organismo quanto na medula espinhal, e sua expressão é alterada em diferentes tipos de patologias envolvendo dor/nocicepção, além de serem regulados por fosforilação. Ainda,

dados recentes apontam os receptores desta superfamília como um possível sensor do estado redox intracelular, podendo ter sua função modulada por agentes oxidantes e redutores (Calixto et al., 2005; Tominaga e Caterina, 2004).

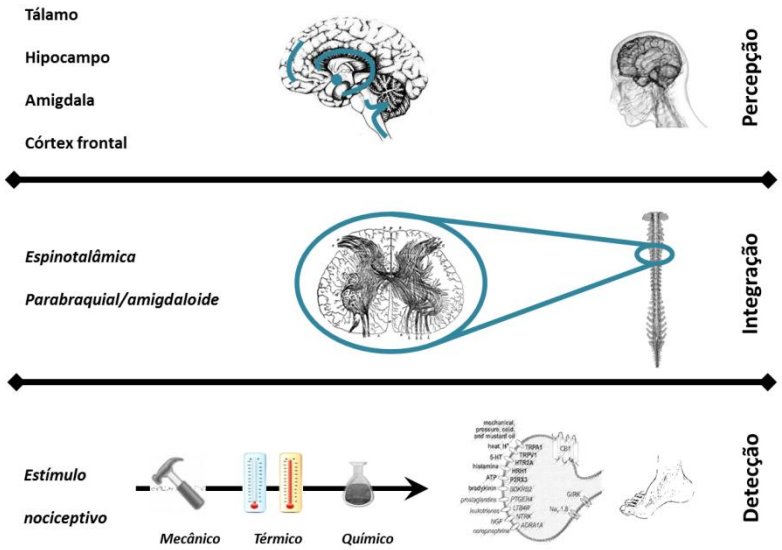

Figura 1: Descrição do processamento geral do estímulo nociceptivo. Representação esquemática da detecção periférica da estimulação nociceptiva, da integração e transmissão do estímulo através da medula espinhal e percepção e reconhecimento deste em diferentes centros supra-espinhais.

1.2. Receptores de potencial transitório - TRP

1.2.1. Família TRP

Os receptores de potencial transitório foram descritos pela primeira vez na década de 1960 por Cosens e Manning (1969), que descreveram a existência de um receptor mutante nos olhos da *Drosophila meganobaster.*

Este receptor apresentava uma resposta transitória à estimulação luminosa e rápida dessensibilização. Posteriormente, descobriu-se que se tratava de uma proteína de 1275 aminoácidos formadores de um canal permeável a cátions, principalmente cálcio (Montell e Rubin, 1989; Phillips et al., 1992).

Apenas na década de 1990 foi descrito o primeiro homólogo dos TRP's em vertebrados (oócidos de *Xenopus* e camundongos), por Peterson e colaboradores (1995), e posteriormente em humanos, por Wes e colaboradores (1995), sendo então denominado TRPC1 (canônico). Até hoje são conhecidas sete diferentes subfamílias, descritas em 29 vertebrados: C – canônico V – vanilóide, M – melastatina, P – policistina, ML – mucolipina, A – anquirina, N – não sensível a estímulo mecânico C (Harteneck *et al*., 2000; Clapham, 2003).

Atualmente, compreende-se que os receptores de potencial transitório (TRP) são uma família de canais iônicos formados por sete subfamílias, mas com características comuns a todos os receptores: capacidade de permear cátions, principalmente cálcio; formados por seis domínios transmembrana com a região formadora do poro entre as regiões cinco e seis, e a região sensor de voltagem entre as regiões um e quatro; cauda C e N terminal apresentam-se voltadas para o lado intracelular, com caráter fortemente hidrofóbico; a região N terminal apresenta-se formada por uma série de resíduos de anquirina, presente na maioria dos canais TRP. Estes resíduos estão dispostos como tijolos empilhados, formando uma superfície estável e rígida para o acoplamento de diversas proteínas; a região C Terminal apresenta diferentes sítios e também uma região conservada (domínio TRP ou *TRP box*), podendo ou não apresentar sítio de ligação para calmodulina (Strübing *et al*., 2001; Montell *et al*., 2005; Zhu, 2005; Latorre *et al.,* 2009; Gaudet, 2009).

A maioria dos TRPs age como sensores fisiológicos a estímulos externos, sejam estímulos térmicos, químicos (variações de pH e osmolaridade), sonoros ou luminosos. Ainda assim, também são capazes de responder a uma gama de substâncias endógenas, como lipídios, nucleotídeos, produtos do estresse oxidativo e açúcares (Jara-Oseguera et al., 2008). Apesar dessas diferenças, os canais TRP, com exceção do $TRPM_{4/5}$, possuem como função controlar a concentração de cálcio (Ca^{++}) intracelular. Com isso, a ativação destes canais leva à ativação de uma série de enzimas sensíveis a cálcio, como óxido nítrico sintase (NOS) e outras enzimas do tipo quinase, bem como transcrição gênica (Tai et al., 2007). Embora se localizem principalmente na membrana celular, alguns TRP podem ainda ser encontrados em algumas organelas, como o retículo endoplasmático e a mitocôndria (Nelson et al., 2010).

1.2.2. O receptor de potencial transitório vanilóide 1 – TRPV1

Na detecção e transmissão de estímulos dolorosos, destaca-se o receptor de potencial transitório da subfamília vanilóide do subtipo 1 (TRPV1). Este receptor é formado por seis domínios transmembrana e um prolongamento hidrofóbico formador do poro, localizado entre as regiões cinco e seis. Similarmente aos demais receptores TRPs, o TRPV1 forma um homotetrâmero funcional, cujos monômeros contribuem para a formação do poro e seletividade do canal (Figura 1) (Tominaga et al., 2005; Ferrer-Montiel et al., 2004). Sua expressão já foi descrita em diferentes tecidos e tipos celulares, como queratinócitos, mastócitos, folículo capilar, células musculares lisas,

bexiga, fígado, rim, baço e pulmão. Além disso, o TRPV1 é intensamente expresso em diferentes regiões do sistema nervoso, como em todos os gânglios (DRG, trigeminal e vagal) e fibras (tipo C e Aδ - peptidérgicas) sensoriais, no nociceptor e na medula espinhal (Caterina et al., 1997, Szallasi e Blumberg, 1999; Sokabe e Tominaga, 2009)

Nestes dois sítios, (nociceptor e medula espinhal), o TRPV1 atua como agente de reconhecimento e integração dos estímulos nociceptivos. Utilizando diferentes métodos de inativação do TRPV1, tanto por ablação gênica quanto por dessensibilização do receptor, é possível observarem-se alterações na capacidade de percepção de estímulos nocivos, bem como no limiar da dor, reforçando a importância do receptor TRPV1 na detecção da dor (Immke e Gavva, 2006; Zhao et al., 2009; Schumacher, 2010). Reforçando este fato, ensaios farmacológicos demonstram o efeito de antagonistas em ambos os sítios (periférica e centralmente), em modelos animais de diferentes patologias associadas à dor e também em humanos (Gavva et al., 2008).

Em ambos os casos, foi observado que a dessensibilização do receptor com capsaicina é capaz de promover uma diminuição da dor inflamatória e neuropática (Szabó et al., 2005). Nestes processos, sabe-se que a ativação TRPV1 é responsável não só pelo desenvolvimento de dor espontânea, mas também de alguns distúrbios sensoriais da dor apresentados pelos pacientes, como alodínia e hiperalgesia (Knotkova et al., 2008).

Fisiologicamente, o TRPV1 é responsável pelo reconhecimento de várias modalidades de estímulos nocivos, como o aumento de temperatura (>42°C) e a diminuição do pH (< 5.5) (Caterina et al., 1997). Este receptor também pode ser ativado por diferentes lipídios, como a anandamida, o ácido

12-hidroperoxi-eicosatetraenóico (12-HPETE), a N-araquidonoil dopamina (NADA), os metabólitos do ácido linoleico oxidado e os prostanóides (Hwang et al., 2000; Shu et al., 2009; Patwardhan et al., 2010,Jara-Oseguera et al., 2008).

O TRPV1 apresenta 18 resíduos de cisteína em sua sequência primária, o que levou diferentes grupos a pesquisarem modulações oxirredutoras de sua atividade. Neste âmbito, foi observado que agentes redutores como a glutationa e o ditiotreitol (DTT) são capazes de promover uma diminuição do limiar de ativação do receptor a temperaturas. As mutações sítio-dirigidas para a Cys621 identificaram um sítio de modulação redox extracelular, principalmente para agentes redutores (Figura 1) (Suzankova et al., 2006).

Apesar disso, já foi demonstrado que alguns agentes oxidantes, como a cloramina-T, e outros agentes alquilantes também são capazes de promover uma diminuição no limiar de ativação térmico do receptor TRPV1 (Susankova et al., 2006). Dentre as radicais livres produzidas endogenamente, destaca-se o peróxido de hidrogênio (H_2O_2), que parece desencadear uma resposta nociceptiva mediada, ao menos em parte, via sensibilização do TRPV1. Também foi descrito que agentes doadores de óxido nítrico (NO), outra espécie reativa produzida endogenamente, são capazes de promover a ativação direta de neurônios que expressam o TRPV1 *in vitro*. Esta resposta foi completamente abolida por antagonistas seletivos deste receptor e também por sua ablação gênica (Keeble et al., 2009). Além disso, também já foi demonstrado que o NO é capaz de interagir diretamente com dois resíduros de cisteína presentes na alça entre os domínios 5 e 6 (formadores do poro). Este processo de oxidação leva a formação de uma ponte dissulfeto “artificial” alterando a conformação do receptor, induzindo ou facilitando sua ativação.

Apesar disso, esses resultados foram obtidos utilizando uma receptor TRP produzido artificialmente, contituido apenas das partes conservadas entre todos os receptores da família TRP (Miyamoto et al.,2009) A partir desses dados, é possível supor que o receptor TRPV1 também se apresente como um sensor do estado redox celular, explicando a sua participação em diferentes patologias (Figura 1) (Lakhan et al., 2009; Mirshafiey et al., 2008).

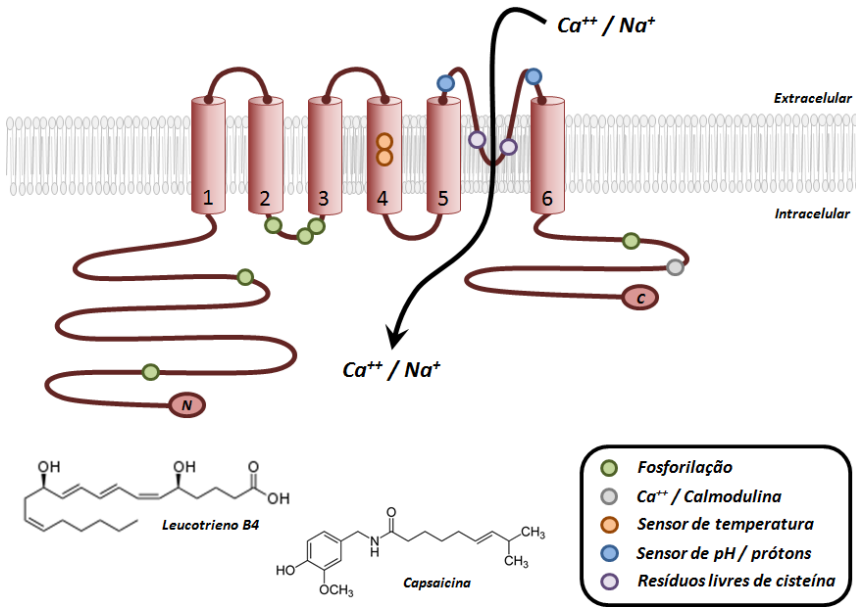

Figura 2: Representação estrutural do receptor TRPV1. Demonstração de diferentes ativadores químicos (leucotrieno B4 e capsaicina), bem como diferentes sítios para o reconhecimento de outros estímulos ativadores (pH e temperatura) e sítios

1.3. Radicais livres e dor

1.3.1. Produção fisiológica de radicais livres

Fisiologicamente, nosso organismo produz de forma contínua uma série de substâncias químicas que apresentam algum átomo com elétron

desemparelhado em sua última camada. Devido a esse desemparelhamento, essas substâncias apresentam elevada reatividade, e por isso são classificadas como radicais livres (RL) (Halliwell e Gutteridge, 2000). A maior fonte produtora de RL's é a mitocôndria, pois aproximadamente 5% do oxigênio molecular (O_2) utilizado na cadeia transportadora de elétrons permanece parcialmente oxidado, gerando assim o radical superóxido ($O_2^{\bullet}$). Além da mitocôndria, outras fontes também são capazes de produzir superóxido, como as enzimas NADPH oxidase (NOX) e a xantina oxidase (Shibata e Kobayashi, 2008; Murphy, 2009).

Como a elevada reatividade dessas substâncias pode ser danosa ao organismo, desenvolvemos uma série de defesas capazes de neutralizar essas substâncias, tanto enzimáticas quanto não enzimáticas (Halliwell e Gutteridge, 2000). As defesas antioxidantes não enzimáticas são compostas em sua maioria por pequenas moléculas capazes de interagir diretamente com as espécies reativas e neutralizar essa reatividade excessiva, protegendo a célula. Dentre estas, destacam-se a vitamina C e vitamina E. Além dessas, as principais defesas antioxidantes não enzimáticas são os compostos tiólicos não proteicos, como a cisteína livre e a glutationa (Halliwell e Gutteridge, 2000). A glutationa é um tripeptídeo formado por glutamato, cisteína e glicina, sintetizada em praticamente todos os tipos celulares através de uma reação de dois passos, limitado pela atividade da enzima γ-glutamil-cisteína-sintetase. Esse tripeptídeo corresponde a aproximadamente 95% do conteúdo total de tióis não proteicos e pode ter a sua síntese modulada positivamente por doadores de cisteína, como a N-acetilcisteína (NAC), e negativamente pelo falso substrato butionina-sulfoxamina (BSO) (Drake et al., 2002). Além de atuar de forma independente, a glutationa também pode ter sua atividade

antioxidante amplificada por uma série de enzimas antioxidantes, juntamente a outros sistemas enzimáticos independentes da glutationa (Moran et al., 2001).

Dentre esses sistemas, destaca-se a glutationa oxidase (GOx) e superóxido dismutase (SOD), capazes de realizar a conversão de $O_2^{\bullet}$ em outra substância menos reativa, o peróxido de hidrogênio (H_2O_2). (Halliwell e Gutteridge, 2000). Como o H_2O_2 ainda apresenta uma reatividade elevada e potencialmente danosa ao organismo, às células também apresentam um mecanismo de neutralização, convertendo o H_2O_2 em O_2 e H_2O. Esta reação de neutralização final fica a cargo principalmente das enzimas catalase (CAT) e glutationa peroxidase (GPx) (Moran *et al.,* 2001).

Outro RL é o óxido nítrico ($NO^{\bullet}$), uma molécula gasosa e extremamente reativa e lipofílica, com meia vida de milissegundos, cujo desemparelhamento eletrônico encontra-se no átomo de nitrogênio (sendo assim uma espécie reativa de nitrogênio - ERN). O $NO^{\bullet}$ é formado pela ação da enzima óxido nítrico sintase (NOS) através da conversão de L-arginina em L-citrulina, com a consequente liberação de $NO^{\bullet}$. Essa reação ocorre na dependência de cofatores como NADPH, tetrahidroxibiopterina (TBH_4) e flavinas (Dinucleotídeo de flavina e adenina – FAD, mononucleotídeo de flavina – FMN). Ainda, a NOS apresenta três isoformas distintas, diferenciadas pela localização, capacidade de síntese e necessidade de cofatores adicionais (Roman e Master, 2006).

A isoforma 1 (neuronal – nNOS) é expressa constitutivamente pelos neurônios (sistema nervoso central – SNC; e periférico - SNP); a isoforma 2 (induzida – iNOS), por micróglia, astrócitos e células do sistema imune mediante estimulação, geralmente por citocinas; e a isoforma 3 (endotelial – eNOS) é expressa constitutivamente no endotélio e na musculatura lisa (Bredt,

1999; Guix et al., 2005). Essas isoformas diferem por sua necessidade de cofatores adicionais: as isoformas 1 e 3 são capazes de produzir grandes quantidades de $NO^{\bullet}$ em espaços mais curtos de tempo, mediante a ativação exercitada por cálcio e calmodulina, enquanto a isoforma 2 independe de ativadores, porém precisa ter sua transcrição gênica induzida por estímulos externos à célula, uma vez que não se encontra expressa constitutivamente. Ainda assim, as isoformas 1 e 3 pode produzir $NO^{\bullet}$ por períodos maiores de tempo (Kamilo et al., 1994).

Devido a essa multiplicidade de isoformas e a presença em diferentes tipos celulares, é controverso justificar a produção de $NO^{\bullet}$ em determinados tecidos por uma única fonte. Por exemplo, no sistema nervoso central, a maior fonte produtora de $NO^{\bullet}$ é a nNOS presente em neurônios. Apesar disso, em determinadas condições, a iNOS expressa pós-estimulação em astrócitos ou pela eNOS do endotélio meningeal pode contribuir significativamente na síntese de NO no sistema nervoso (Lorenc-Koci e Czarnecka, 2005).

Em virtude dessa reatividade e rápida difusão, o $NO^{\bullet}$ consegue reagir de forma inespecífica com resíduos de cisteína de diferentes proteínas (nitrosilação proteica). Essa reação forma um produto mais estável que o $NO^{\bullet}$ livre, mas ainda assim lábil, podendo ser revertida em certas condições, como na presença de antioxidantes. Esta alteração pode levar até mesmo a uma alteração da função da proteína alvo (Derakhshan et al., 2007). Além de reagir com resíduos de cisteína, o $NO^{\bullet}$ também pode reagir com $O_2^{\bullet}$, formando outra espécie reativa de nitrogênio, o peroxinitrito ($ONOO^{\bullet}$) (Beckman et al., 1990). Similarmente ao $NO^{\bullet}$, o peroxinitrito também se apresenta como gás em condições fisiológicas, com elevada reatividade e lipofilicidade. Ao contrário do

$NO^{\bullet}$, o peroxinitrito demonstra uma elevada afinidade por resíduos de diferentes aminoácidos, especialmente pela tirosina. Tal reação (nitração) também ocorre de forma inespecífica, podendo ou não alterar a funcionalidade da proteína alvo. Apesar disso, ao contrário da nitrosilação, a elevada reatividade do peroxinitrito forma um produto altamente estável e irreversível, a 3-nitrotirosina (3-NT) (Pacher et al., 2007).

Diferentemente das demais RL's que apresentam um sistema enzimático especializado para sua neutralização, o $NO^{\bullet}$ é neutralizado espontaneamente por sua redução a nitrato (NO_3) e nitrito (NO_2), permanecendo em equilíbrio entre estas duas formas (NO_2/NO_3 – NOx). Além disso, o $NO^{\bullet}$ também pode ser neutralizado por compostos tiólicos, como a glutationa e a cisteína livre, formando os nitroso-tióis, formas mais estáveis em condições fisiológicas (Martínez-Ruiz et al., 2007).

Quando ocorre algum desequilíbrio entre a produção de RL’s e as defesas antioxidantes do organismo, pode haver a oxidação descontrolada de diferentes tipos de biomoléculas, como proteínas, lipídios e ácidos nucléicos. Este processo faz com que ocorra alteração ou perda total da funcionalidade dessas biomoléculas, com consequente dano celular. A este processo caracterizamos como estado de estresse oxidativo (Halliwell e Gutteridge, 2000; Starkov, 2008). Tal desequilíbrio já foi observado em diferentes estados patológicos, como artrite reumatoide, lesão por isquemia-reperfusão e diabetes. Nestes quadros, as RL’s foram apontadas como mecanismos de lesão celular, bem como mantenedoras do processo patológico (Kundu et al., 2012; El Boghdady et al., 2012; Veselinovic et al., 2014). Devido a essa elevada importância no organismo, o uso clínico de diferentes antioxidantes já foi

indicado como tratamento ou terapia adjuvante para diferentes condições patológicas. Diferentes testes clínicos já apontaram para o benefício da utilização da NAC em diferentes patologias de origem inflamatória, neurodegenerativa e dolorosa (Gracey et al., 1969; Perez et al., 2003).

Um dos principais processos em que vem se observando o desenvolvimento de estresse oxidativo são eventos dolorosos. A participação de RLs e do estresse oxidativo na produção da dor foi inicialmente evidenciada em estudos *in vitro*, nos quais o H_2O_2 e $O_2^{\bullet}$ estimularam nociceptores cardíacos e cutâneos, especialmente após isquemia ou aplicação de mediadores inflamatórios (Kress *et al.,* 1995; Huang *et al.,* 1995). Também, estudos *in vivo* demonstraram que várias condições patológicas cujo principal sintoma é a dor são acompanhadas do desenvolvimento de estresse oxidativo em diferentes tecidos (Wang *et al.,* 2004). Ademais, foi evidenciado que o tratamento com substâncias antioxidantes promove uma melhora do quadro clínico doloroso em humanos, bem como em modelos animais de nocicepção (Perez *et al.,* 2003; Hacimuftuoglu *et al.,* 2006). Nestes trabalhos, foi demonstrado que o tratamento sistêmico com antioxidantes reduz a nocicepção produzida por diferentes estímulos nociceptivos, como agentes inflamatórios e lesões neuropáticas (Guedes et al., 2006; Kim et al., 2004).

Um dos antioxidantes mais utilizados atualmente é a vitamina E. Este nome refere-se a uma classe de compostos (tocoferóis) lipofílicos com atividade antioxidante presentes em diferentes alimentos (Govind et al., 2004; Yamauchi, 2007; Brigelius-Flohé , 2009;). Sua atividade antioxidante já foi descrita em diferentes sistemas, mas principalmente de compartimentos lipídicos celulares, como membranas. Clinicamente, o uso da vit-E já foi

indicado para diversos quadros patológicos, principalmente relacionados a desordens inflamatórias crônicas, como algumas respostas de hipersensibilidade, inflamação nas vias respiratórias e diferentes tipos de artrites (reumatoide e osteoartrite) (Cook-Mills, 2013). Nessas patologias já foi descrito um amplo aumento nos marcadores de estresse oxidativo em amostras de soro, os quais já foram apontados como responsáveis pela manutenção de diferentes sintomas apresentados no curso da doença (Kheir-Eldin et al., 1992). Apesar disso, ainda existem relatos conflitantes sobre a sua real eficácia nesses quadros, bem como de seus efeitos e mecanismos (Canter et al., 2007; Aryaeian et al., 2009). Por isso, é necessário esclarecer os reais efeitos desse antioxidante e seus possíveis mecanismos anti-inflamatórios.

Da mesma forma, evidências sugerem o efeito dual de algumas radicais livres na nocicepção, e o principal exemplo disso é o $NO^{\bullet}$. Diversos trabalhos já relataram a importância da produção de $NO^{\bullet}$ para a atividade analgésica de alguns fármacos, como a morfina, e também que a administração de doadores de $NO^{\bullet}$ promovem efeito antinociceptivo (Ferreira et al., 1991). Contudo, no SNC, o $NO^{\bullet}$ é reconhecido como uma molécula classicamente pró-nociceptiva. Esse fato é suportado por evidências mostrando que a administração intratecal de doadores de NO é capaz de induzir nocicepção, bem como há também evidências de que inibidores da NOS apresentam efeito antinociceptivo (Meller et al., 1992; Chen et al., 2010). Classicamente, este efeito é atribuído às ações do $NO^{\bullet}$ junto ao sistema glutamatérgico, mediante modulação do receptor N-metil-D-aspartato (NMDA), ou através da ativação da enzima guanilato ciclase (Cury et al., 2011).

No entanto, já foi demonstrado que a administração exógena e periférica de doadores de óxido nítrico é capaz de desencadear nocicepção em roedores de forma dependente dos TRPA1 e TRPV1 (Miyamoto et al., 2009). Além disso, esses mesmos doadores são capazes de desencadear influxo de cálcio em células do gânglio da raiz dorsal (DRG) de camundongos. Embora esse influxo seja prevenido pela incubação prévia ao estímulo com antagonistas de ambos os receptores (HC030031 e SB366791, respectivamente), apenas a ablação gênica do receptor TRPV1, mas não do TRPA1, foi capaz de abolir essa resposta.

Mesmo assim, não existem evidências de que a produção de RL's geradas endogenamente, como o óxido nítrico, sejam capazes de desencadear e/ou manter uma resposta álgica mediada através da ativação direta do receptor TRPV1. Também, não existem evidências que indiquem o mecanismo exato de ação analgésica/antinociceptiva dos antioxidantes em humanos e camundongos. Dessa forma, investigar estes mecanismos ainda se mostra necessário, não somente para melhor entendimento da patofisiologia da dor, mas também para apontar novos alvos e terapias eficazes para o seu tratamento.

2. OBJETIVOS

2.1. Objetivo geral

Investigar o efeito antinociceptivo da N-acetilcisteína (NAC) e vitamina E (vit-E) em diferentes modelos de nocicepção, bem como possíveis mecanismos de ação.

2.2. Objetivos específicos

1. Avaliar o possível efeito antinociceptivo de um tiol não proteico exógeno (NAC) administrado por várias vias em diferentes testes de nocicepção;
2. Averiguar se a nocicepção periférica induz estresse oxidativo na medula espinhal de camundongos;
3. Investigar se a depleção dos níveis de tióis não proteicos endógenos altera a sensibilidade nociceptiva dos camundongos;
4. Verificar se o NO poderia alterar os níveis de tióis não proteicos e a transmissão espinal da nocicepção via estimulação do receptor TRPV1;
5. 5. Avaliar o efeito antinociceptivo da vit-E em modelo de inflamação crônica em ratos;
6. Averiguar o possível efeito da vit-E sobre parâmetros inflamatórios crônicos, em ratos;
7. Investigar o efeito antioxidante da vit-E em ratos submetidos a processo inflamatório crônico.

3. ARTIGO 1

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Spinal Levels of NonProtein Thiols Are Related to Nociception in Mice

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Abstract: Oxidative stress markers are thought to be related to nociception. Because thiolic compounds are important antioxidants, we investigated the relationship between thiols, endogenous or exogenous, and nociception. Systemic or spinal, but not peripheral, administration of the exogenous thiolic compound N-acetyl-L-cysteine (NAC) reduced nociception induced by intraplantar capsaicin injection. Moreover, we detected an increase in lipid peroxidation and 3-nitrotyrosine and a decrease in nonprotein thiolic levels in the lumbar spinal cord of capsaicin-injected animals. All these effects were prevented by NAC treatment (i.p. and i.t.). Our findings confirm a role for the spinal cord in NAC actions because systemic NAC administration also reduced the nociception trigged by intrathecal injection of capsaicin. Moreover, adjuvant-induced arthritis, but not paw incision, also -decreases nonprotein thiol levels in the spinal cord. Similarly, NAC produced antinociception in adjuvant-treated animals, but not in paw-incised animals. Finally, we investigated the role of endogenous thiol compounds in the nociceptive process administrating buthionine-suphoxamine (BSO), an inhibitor of glutathione-synthesis. Intrathecal BSO treatment decreased nonprotein thiol levels in the spinal cord, as well as induced mechanical allodynia and chemical and thermal hyperalgesia. In conclusion, our results indicate a critical role for nonprotein thiols in nociception at the level of the spinal cord.

Perspective: *The results presented here indicate that the loss of nonprotein thiols in the spinal cord is involved in pain development. Therefore, the administration of thiolic compounds or other strategies allow thiol levels to be maintained and could be a beneficial action in the therapy of painful conditions.*

Key words: Pain, capsaicin, NAC, TBARS, thiol, BSO.

During normal metabolic processes, small amounts of reactive species are produced. Due to their reactive nature, cellular levels of these species are tightly controlled by endogenous antioxidant systems. Imbalance between reactive species and antioxidant systems results in tissue injury from reactive species interactions with biomolecules, and therefore a state of oxidative stress.[17]

Recent in vivo studies have demonstrated the involvement of oxidative stress in pain processes. For example, oxidative stress development has been shown in neuropathic and inflammatory pain models.[26,37] In addition, studies have shown that exogenous antioxidant compounds, such as PBN (N-*tert*-Butyl-α-phenylnitrone), α-tocopherol, TEMPOL (4-Hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl), and N-acetylcysteine, produce antinociceptive effects.[14,16,20] An antinociceptive effect of antioxidants administered intrathecally (i.t.) has previously been demonstrated,[19] and this effect was attributed to inhibition of central sensitization.

Thiolic compounds (substances with free Sulfur-H groups) are some of the most important endogenous

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antioxidant defenses in mammalian organisms. Among these compounds, metallothionein and glutathione (GSH) are the major protein and nonprotein thiolic compounds, respectively.[25] GSH is the most abundant nonprotein thiolic compound intracellularly, as well as in body fluids such as cerebrospinal fluid, where its concentration reaches 1 μM. Furthermore, 97% of GSH exists in the reduced form.[8] GSH is synthesized from glutamate, cysteine and glycine through the activity of γ-glutamil-cysteine synthase and glutathione synthetase. GSH synthesis can be increased by cysteine analogs such as N-acetyl-L-cysteine (NAC), or decreased by γ-glutamil-cysteine synthetase inhibitors such as buthionine-sulphoxamine (BSO).[1,13] Because thiol compounds are important for preventing oxidative stress, the present study aimed to evaluate the role of endogenous and exogenous thiols in pain models.

Methods

Animals

Three-month-old male and female albino Swiss mice (25–35 g) bred in our animal house were used in this study. Animals were housed at a controlled temperature (22 $\pm$ 2 °C) with a 12 hour light/dark cycle, and were provided standard lab chow and tap water ad libitum. The animals were habituated to the experimental room for at least 1 hour before experiments. Each animal was used for only 1 experiment. The experiments reported in this study were carried out in accordance with current ethical guidelines for investigation of experimental pain in conscious animals.[42]

Drug Treatment

To increase endogenous thiol levels, mice were treated with NAC diluted in saline and administered intraperitoneally (i.p.; 30–300 mg/kg) or phosphate-buffered saline (PBS, pH 7.4) administered i.t. (3–300 nmol/site) and intraplantarly (i.pl; 100 nmol/site). The doses used were based on pilot experiments. The procedure for i.t. drug administration was based on the technique described elsewhere.[18] Briefly, the intervertebral space between L5 and L6 was punctured directly using a 28-gauge needle attached to a Hamilton microsyringe. A total volume of 5 μL was administered intratecally. Mice were not anesthetized during these procedures. A tail flick was used as an indication that the needle had penetrated the dura.

To induce decreased thiol levels, a separate group of animals was injected i.t. with BSO (3.2 μmol/site)[1] or its vehicle (PBS). Twenty-four hours after the BSO treatment, the development of mechanical hyperalgesia and allodynia and thermal and chemical hyperalgesia were determined as described below.

Capsaicin Test

The peripheral capsaicin test was carried out as previously described.[12,30] Animals were habituated to the observation location, consisting of a glass chamber, for at least 30 minutes before the experiment. Following habituation, .3 or 3 nmol (submaximal and maximal doses, respectively) of capsaicin was injected into the right hind paw of mice, and the total time spent licking the injected paw during 5 minutes was measured. The amount of time spent licking was considered a measure of nociception. For the spinal capsaicin test, capsaicin was injected i.t. with a dose of .2 nmol/site, as previously described.[31,38] In this test, the total time spent licking the hind paws and tail during the 5 minutes following injection was determined and considered a measure of nociception. The proper vehicle (.15% ethanol in saline for i.pl test and PBS for the i.t. test) was prepared and used as a control treatment. Vehicle administrations did not evoke nociceptive behavior themselves.

Mechanical Sensibility

The measurement of mechanical sensibility was performed as previously described.[11] Mice were habituated to wire-mesh-bottom cages 1 day before the experiment and for 2 hours before the start of the experiment. Static allodynia and hyperalgesia was tested by touching 10 times the plantar surface of the hind paw with a von Frey hair of .05 g (for allodynia) and 2 g (for hyperalgesia). The paw-withdrawal frequency was measured and considered as a measure of allodynia.

Thermal Hyperalgesia

The development of thermal hyperalgesia was evaluated according to with minor modifications.[9] Briefly, before drug administration, the animal was gently immobilized, and the right hind paw was immersed in a water bath at 48 $\pm$ 1°C in order to determine the basal withdrawal latency. The latency to withdrawing the paw from the hot bath was recorded manually with a stopwatch.

Inflammatory Pain Models

To investigate the effect of NAC in more clinically relevant pain models, we tested NAC on allodynia developed by 2 distinct processes. First, we evaluated the effect of NAC in Complete Freund Adjuvant (CFA)-induced arthritis. To induce inflammation, animals were slightly anesthetised with halothane and 20 μL of CFA (.6% suspension of heat-killed *Mycobacterium tuberculosis* in liquid paraffin) was injected into the right hind paw. Forty-eight hours later, the development of mechanical allodynia was evaluated as previously described following NAC administration.[4,11,12]

The second model we used was the postsurgical pain model. This test was carried out according to a procedure described in rats[6] and adapted to mice.[29] Mice were anesthetized with 2% halothane via a nose cone. After antiseptic preparation of the right hind paw with a 10% povidone-iodine solution, a 5-mm longitudinal incision was made with a number 11 blade through the skin and fascia of the plantar foot. The incision started 2 mm from the proximal edge of the heel and extended towards the toes. The underlying muscle was elevated with curved forceps, leaving the muscle connected to the bone. The skin was closed with a single mattress

suture with 6.0 nylon. The treatment was conducted after confirming the development of mechanical allodynia, as described above.

Estimations of Thiol Levels and Lipid Peroxidation

After the algogenic tests, animals were sacrificed with halotane and the lumbar portion of the spinal cord (L1 to L6) was collected for biochemical assays. To estimate total and nonprotein thiol contained in the sample, tissue was homogenized in 1.0 mL of .02 M EDTA and maintained on ice for the remainder of the procedure. The homogenate was centrifuged at 4,000 g for 15 minutes at 4°C. The supernatant was then divided into 2 aliquots of 300 μL (A and B). Aliquot A was used to determine total thiol content. Aliquot B was incubated with 50 μL of 50% TCA at 4°C for 60 minutes. Following incubation, aliquot B was centrifuged at 12,000 g for 5 minutes at 4°C, and the supernatant was collected to determine the nonprotein thiol content in the sample. To determine total thiol content, 200 μL of Tris/HCl (200 mM, pH 8.9) and 20 μL of DTNB (2.5 mM) were added to a 100 μL sample in a microplate and incubated at room temperature for 5 minutes.[32] Protein thiol content was calculated by subtracting the values of nonprotein thiols from total thiols. The color of the solution resulting from the reaction was measured at 405 nm with a FisherBiotech Microkinetics Reader BT 2000 (Pittsburgh, PA). Cysteine was used as a standard to determine the thiol (SH) content in the samples.

To evaluate lipid peroxidation induced by nociceptive stimuli, tissue samples were collected after the test, homogenized in 1.5 mL buthylated hydroxytoluene (BHT; 40 nM) in PBS, and maintained on ice for the remainder of the procedure. The thiobarbituric acid reactive species (TBARS) reaction was performed as described elsewhere.[3,28] The solution color resulting from the reaction was measured at 535 nm using a Hitachi U-2001 Spectrophotometre (Sataiama, Japan). Malonyldialdehyde (MDA) was used as a standard to determine the MDA level in the sample.

Both the TBARS and thiol contents of the samples were corrected for the protein contents of the samples. Protein was measured using Comassie-Blue dye[5] using bovine serum albumin as a standard.

Slot Blot Assay for 3-Nitrotyrosine

Peroxinitrite is one of the most reactive nitrogen species formed during oxidative stress and can attack proteins and alter its function, producing 3-Nitrotyrosine (3-NT). Thus, 3-NT levels were measured[19] after capsaicin or vehicle-paw injection in lumbar spinal cord samples as a marker of reactive specie production. Briefly, 5 μL of sample were added to 5 μL of 12% SDS (sodium dodecyl sulphate, Sigma) and 5 μL of Laemmli buffer (.125 M Tris, pH 6.8, 4% SDS, and 20% glycerol) and incubated for 20 minutes at room temperature. The resulting solution was loaded in a nitrocellulose membrane under vacuum using a slot blot apparatus. The membrane was blocked in blocking buffer (3% bovine serum albumin) for 1 hour and incubated with a 1:2,000 dilution of anti-3-NT polyclonal antibody in PBS (.05 mM, pH 7.4) containing .01% sodium azide and .2% Tween 20 for 1 hour. The membrane was washed 3 times in PBS and was incubated for 1 hour with an antirabbit IgG alkaline phosphatase secondary antibody diluted in PBS (1:8,000). Blots were dried, scanned with Adobe Photoshop, and quantified with Scion Image (PC version of Macintosh compatible NIH image). The 3-NT blot had a faint background that was corrected in image analysis.

Drugs and Reagents

Algogenic Test

Capsaicin and Complete Feund' Adjuvante (CFA) were purchased from Sigma (St Louis, MO). Capsaicin was dissolved in 90% ethanol and 10% Tween 80, and then diluted to the appropriate concentration in saline for intraplantar administration or PBS for i.t. administration.

Mice Treatment

BSO (L-buthionine-[S,R]-sulfoximine) was also purchased from Sigma and dissolved in PBS for stock solution and administration. NAC was purchased from Zambon (São Paulo, Brazil, Fluimicil®) and diluted in saline for systemical administration and in PBS for intratecal and intraplantar administration. SB-366791 was purchased from Sigma and diluted in 10% ethanol for stock solution and diluted in PBS for intraplantar administration.

Oxidative Stress Experiments

Thiobarbituric acid (TBA; Fluka, Buchs, Switzerland) and Tricloroacetic acid (TCA, from VETEC, Rio de Janeiro, Brazil) were diluted in water prior use. Buthylated hydroxytoluene (BHT) was also purchased form VETEC and was dissolved in ethanol and then diluted in PBS (pH 7.4) to the desired concentration (40 nM). Anti-3-NT polyclonal antibody and DTNB (5,5'-dithiobis(2-nitro-benzoic acid)) were purchased form Sigma, and DTNB was prepared in methanol for stock solution and diluted in water for desired concentration (2.5 mM).

Statistical Analysis

Results are expressed as mean ±S.E.M. Statistical analyses were performed using t-tests, or 1-way or 2-way analysis of variance (ANOVA) with Student-Newman-Keuls (SNK) post hoc tests when necessary. The level of significance was set at $P < .05$. The percent of maximal inhibition was determined for each experimental group using the following formula: % Inhibition = 100 − [(Experiment × 100)/Control].

Results

Effect Of Exogenous NonProtein Thiol Administration on Acute Nociception

Administration of NAC (30–300 mg/kg, i.p.) resulted in a significant antinociceptive effect in the intraplantar capsaicin test. Specifically, at the timepoint of maximal effect following drug administration, NAC inhibited the

nociceptive response to 46 ± 9% in comparison to vehicle-treated animals (Fig 1B). The antinociceptive effect of NAC (100 mg/kg, i.p.) was not seen at .5 or 2 hours, but was significant at 1 hour, thus indicating a late onset and short-lived antinociceptive effect (Fig 1A). At 100 mg/kg, no alteration in the rotarod test was found in either the time of first fall (vehicle: 126.5 ± 40.1 seconds; NAC: 115.3 ± 37.1 seconds) or the number of falls (vehicle: 1.0. ± .1; NAC: 1.2 ± .2). It indicates that the antinociception was not due to motor impairement. NAC administered i.t. (300 nmol/site) also produced a significant antinociceptive effect at both 30 and 60 minutes after treatment (Fig 1C). Intrathecal administration of NAC i.t. was antinociceptive at doses of 10 to 300 nmol/site, producing 59 ± 7% inhibition compared with vehicle-treated animals at 300 nmol/site (Fig 1D). However, intraplantar (i.pl) administration of NAC (300 nmol/paw) 10 minutes before the i.pl injection of capsaicin (3 nmol/paw) produced no antinociceptive effect, while pretreatment with SB-366791 (1 nmol/site, i.pl.), a selective TRPV1 receptor antagonist used as a positive control, significantly reduced the capsaicin-induced nociception (Fig 2A).

The antinociceptive effect of systemic and i.t. administration of NAC and the lack of a peripheral effect suggest that the spinal cord may be the primary site responsible for NAC antinociceptive action. To further investigate this possibility, NAC was administered i.p. (100 mg/kg) and capsaicin was injected directly into the spinal cord (.2 nmol/site). In this protocol, a 1 hour pretreatment with NAC also produced a significant antinociceptive effect (66 ± 5% inhibition; Fig 2B).

Effect of Acute Nociception on Spinal Levels of Thiols and Oxidative Stress

NAC treatment increased total thiol levels (65.3 ± 20.3% for i.p. and 119.1 ± 35.2 for i.t.) and nonprotein thiol levels (84.1 ± 32.9% for i.p. and 121.1 ± 33.9% for i.t.) in the spinal cord after treatment (100 mg/kg, i.p. and 300 nmol, i.t.). Capsaicin injection (i.pl; 3 nmol/paw) produced no change in spinal total thiol levels (data not shown), but induced a decrease in nonprotein thiol levels (47.8 ± 9.5% for i.p. and 37.7 ± 6.3% for i.t. vehicle-treated). In this study, NAC was also able to prevent nonprotein thiol loss in the spinal cord (Figs 3A and 3B).

Because this reduction in thiol level may be due to oxidative stress, we assessed lipid peroxidation by measuring thiobarbituric acid reactive species (TBARS). Intraplantar injection of capsaicin increased lipid peroxidation compared with vehicle-treated animals (58.1 ± 19.2% for i.p. and 45.7 ± 6.3% for i.t. injection). Pretreatment with NAC (100 mg/kg, i.p.; Fig 3C, or 300 nmol/site, i.t.; Fig 3D) abolished the rise in lipid peroxidation induced by capsaicin.

To confirm the oxidative stress induced by capsaicin, we measured the spinal levels of 3-nitrotyrosine (3-NT), a specific product formed by the reaction of the reactive specie peroxynitrite with tyrosine residues of proteins. After capsaicin injection, an increase was observed in the levels of 3-NT in the lumbar spinal cord (54.7 ± 5.3). The systemic treatment with NAC (100 mg/kg, i.p.) is also capable to fully prevent the rise of 3-NT levels in capsaicin-injected, but not in vehicle-injected animals (Fig 3E).

Effect of BSO Intrathecal Treatment on Nociceptive Behavior

After the effect of exogenous thiol-compound treatment was evaluated and its critical role of the spinal cord was characterized, we investigated the participation of the spinal cord endogenous thiol system in nociceptive processing. To do so, mice were treated with the γ-glutamilcysteine synthetase inhibitor BSO (3.2 nmol/site, i.t.). Twenty-four hours after BSO treatment, we

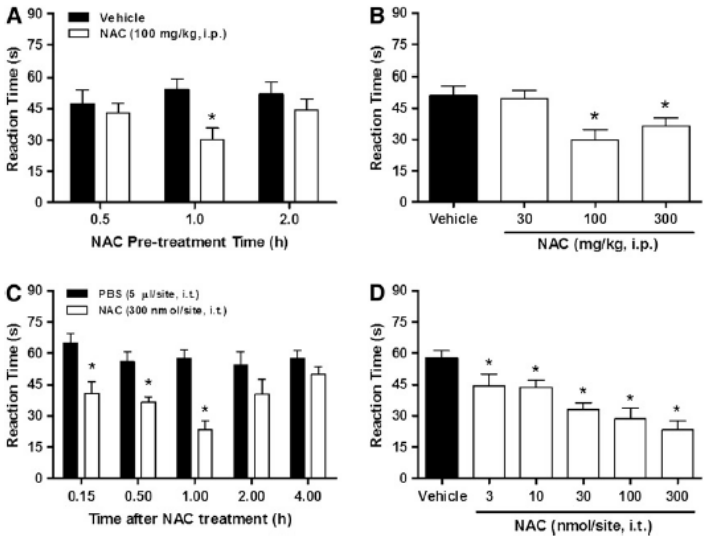

Figure 1. Time- (**A and C**) and dose- (**B and D**) dependent antinociceptive responses to intraplantar capsaicin injection (3 nmol/paw) (n = 6–8). Either vehicle or NAC (N-acetylcysteine) were administered to mice by either i.p. (**A and C**) or i.t. (intrathecal; **C and D**) injection. Data are expressed as means + S.E.M. *$P < .05$ based on 2-way (**A, C**) or 1-way ANOVA (**B, C**) and SNK post hoc test.

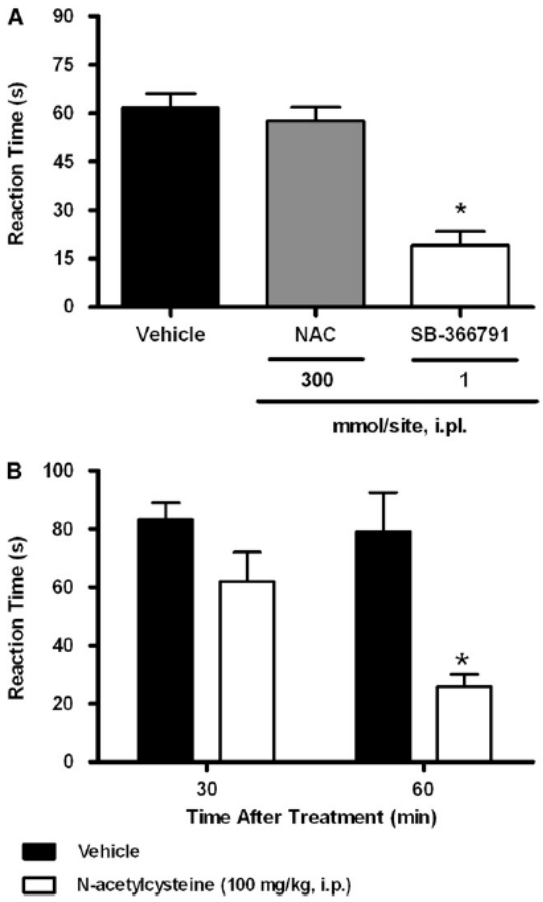

Figure 2. **(A)** Antinociceptive effect of SB-366791 and lack of antinociceptive effect of NAC (N-acetylcysteine) when administered i.pl 10 minutes before the intraplantar capsaicin test (3 nmol/paw) (n = 6). **(B)** Antinociceptive effect of i.p. administration of NAC on the nociceptive response produced by i.t. capsaicin injection (n = 6). Data are expressed as mean + S.E.M. $*P < .05$, based on 2-way ANOVA and SNK post hoc test.

found a decrease in thiols levels in the lumbar spinal cord (Fig 4A). Moreover, BSO treatment also induced mechanical allodynia and hyperalgesia (6.6-fold and 1.5 increase in withdrawal frequency, respectivelly; Figs 4B and 4C) and thermal hyperalgesia (4.8-fold decrease in withdrawal latency; Fig 4D). BSO-treated animals also displayed chemical hyperalgesia, with a 2.2-fold increase in nociception as when stimulated with a submaximum dose of capsaicin (.3 nmol/paw; Fig 4E).

Effect of Exogenous NonProtein Thiol Administration in Clinically Relevant Pain Models

To determine the role of nonprotein thiols in clinically relevant pain models, we investigated the level of thiols and the effect of exogenous thiols in arthritis and postsurgical pain models. NAC produced antinociceptive effect in CFA-induced arthritis, showing antiallodynic effect at .5 to 4 hours after its intraperitoneal (100 mg/kg; 47.0 ± 5.9% of inhibition) or intrathecal (300 nmol/site; 41.4 ± 4.3% of inhibition) treatment (Figs 5A and 5B). Similarly to capsaicin test, no effect was observed in the CFA-model after intraplantar NAC administration (300 nmol/paw, Fig 5C). In this model, the total and nonprotein thiol levels in the spinal cord were also estimated. Similar to the capsaicin test, CFA injection decreased spinal cord nonprotein thiol levels, but not protein thiol levels, and this effect was reversed with NAC treatment (Fig 5E).

NAC was also evaluated in a model of postincisional pain. However, NAC treatment produced neither anti-allodynic effects (Fig 5D) nor altered nonprotein thiol levels in the spinal cords of incised animals (Fig 5F).

Discussion

Thiols are compounds containing SH groups, are mostly found in cysteine, and are present in several high- and low-molecular weight biomolecules. In protein, thiols can provide structure or act as regulatory centers. In nonprotein substances, thiols such as glutathione play a major antioxidant defence role in cells. The importance of nonprotein thiols in many pathological states, such as Alzheimer's disease, Parkinson's disease, and epilepsy, is well known.[22,34,35] However, the role of thiols in pain is poorly understood. Our study demonstrates the importance of both exogenous (through NAC administration) and endogenous (through BSO treatment) nonprotein thiols in nociception.

The antinociceptive effect of NAC was initially evaluated in the capsaicin test, a well-characterized model of neurogenic pain.[15,33,39] The decision to use the capsaicin test was made by its well-established mechanisms and the previously knowledge of the events triggered by capsaicin stimulation.[12,30,31,38,39] Besides, this test was previously demonstrated to be a good model to evaluate the antinociceptive effect of antioxidants.[21] NAC was able to induce antinociception in the capsaicin test without motor impairment when administered systemically. These findings are consistent with previous findings of NAC producing antinociception in the neurogenic phase of the formalin test in mice.[16] These data support the hypothesis that reactive species are involved in neurogenic pain.

Interestingly, we found that NAC produced increased antinociception in the central capsaicin test, which indicates that NAC acts centrally. We next assessed whether NAC administered i.t. would enhance the antinociceptive effect in the i.pl. capsaicin test. In this model, NAC demonstrated a slight increase in effectiveness. To exclude a possible peripheral action of NAC, it was administered peripherally (i.pl.), and produced no antinociceptive effect. These results suggest that the major site of action of NAC is in the spinal cord. Despite its limitated capacity to cross the blood-brain barrier when administrated systemically, NAC are able to reach the spinal cord.[10] This finding explains the antinociceptive effect produced by NAC systemic administration. The importance of the

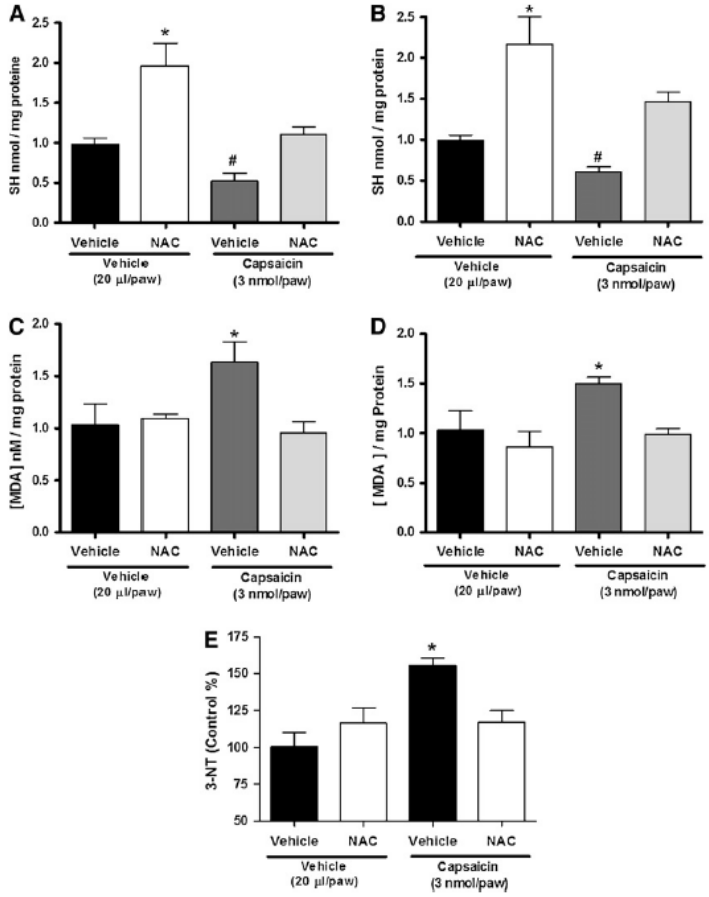

Figure 3. Markers of oxidative stress levels measured 5 minutes after intraplantar capsaicin injection (3 nmol/paw). Nonprotein thiol content in spinal cord samples after i.p. **(A)** or i.t. **(B)** NAC (N-acetylcysteine) treatment (n = 4–6); Lipid peroxidation in spinal cord after i.p. **(C)** or i.t. **(D)** NAC treatment (n = 4–6) and 3-nitrotyrosine (3-NT) levels **(E)** in spinal cord after NAC treatment (100 mg/kg, i.p.) (n = 4). Data are expressed as mean + SEM. *$P < .05$, compared with vehicle pretreated mice (black bars), based on 1-way ANOVA and SNK post hoc. Nonprotein thiol and lipid peroxidation levels are represented as free SH levels and -MDA levels, respectively.

spinal cord in the antinociceptive effects of antioxidants has been suggested[19] when they showed that PBN has a greater antinociceptive effect when administered i.t. compared with subcutaneous administration.

The proposal that reactive species are important in spinal-cord processing is strengthened by the finding that harmful stimulation induces a reduction in nonprotein thiols levels, together with an increase in the lipid peroxidation. These results demonstrate the importance of nonprotein thiols at the level of the spinal cord in pain transmission. The reduction in nonprotein thiol levels in capsaicin-treated animals may be explained by nonprotein thiol consumption by free radicals generated during nociceptive transmission. As free radicals are very difficult to be directly measured in vivo,[27] we detected their production indirectly by the measurement of 3-NT levels in lumbar spinal cord. During oxidative stress, the combination of nitric oxide (NO°) and superoxide radical (O_2°) form peroxynitrite ($ONOO^{-}$), one of the most reactive species formed in mammalian organism. Peroxynitrite atacks tyrosine residues of proteins to generate 3-NT, Thus, nitric oxide, superoxide radical and peroxynitrite seems to be produced in spinal cord during nociceptive transmission since intraplantar capsaicin injection lead to an increase in the levels of 3-NT in lumbar spinal cord.

In either cerebrospinal fluid or the intracellular medium, thiol compounds, especially glutathione, are responsible for neutralizing several types of reactive species, such as nitric oxide and superoxide. At spinal cord, the major excitatory neurotransmitter is glutamate, which leads to activation of NMDA receptors and increased intercellular Ca^{++} levels. This Ca^{++} rise increases the activity of several free radical-generating enzymes, such as nitric oxide synthase (NOS) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.[2] Accordingly, both spinal NMDA receptors and NOS participate in nociception induced by i.pl. capsaicin.[31] These events may explain the decrease in nonprotein thiol and the increase in 3-NT levels observed after

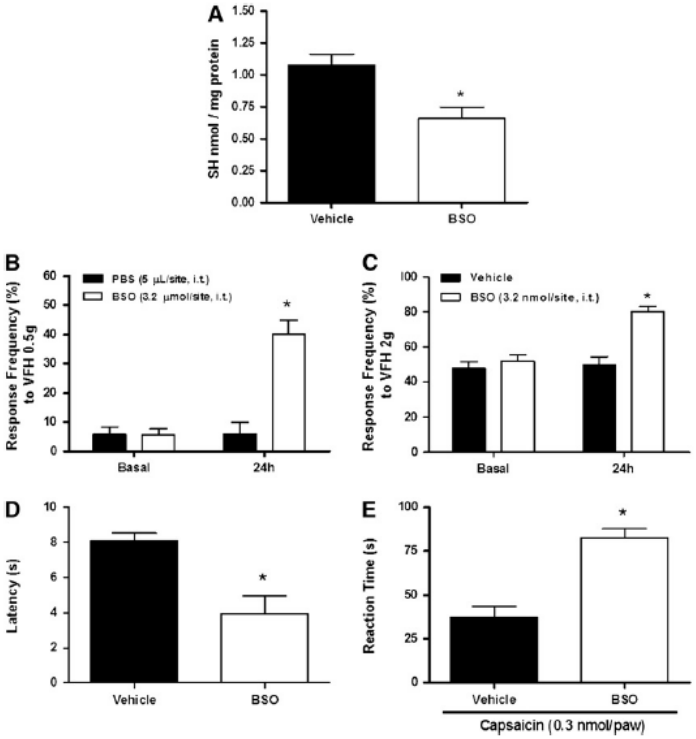

Figure 4. Effect of BSO (buthionine-sulfoxamine)-induced (3.2 nmol/site, i.t.) treatment in mice (n = 5). **(A)** Nonprotein thiol levels after 24 hours after BSO treatment. Same treatment induced mechanical allodynia **(B)** and hyperalgesia **(C)** to 10 stimulations with von Frey Hair (VFH). Despite it, it was also observed the development of thermal hyperalgesia **(D)** at 48 °C water bath and chemical hyperalgesia **(E)** in the capsaicin test (.3 nmol/paw). Data are expressed as mean + SEM. * $P < .05$, based on Student t-test.

capsaicin stimulation and the ability of NAC to reverse these events.

Once generated, there are some possibilities to explain how free radicals might increase pain transmission. It has been demonstrated that oxygen and nitrogen reactive species can decrease the activities of glutamate transporters and increase the activation of vanilloid and NMDA receptors trough 3-NT formation.[7,36,40] In addition, GSH and cysteine have been shown to decrease substance P binding to NK1 receptors on striatal membranes.[23] All of these mechanisms could contribute to increased pain transmission in the spinal cord. Therefore, by increasing the antioxidant defenses through nonprotein thiols, it may be possible to reduce central sensitization and inhibit pain transmission.

In the spinal cord, there are several types of nonprotein thiolic compounds, such as cysteine. The major nonprotein thiolic compound, both intracellularly and extracellularly, is GSH. Therefore, we investigated the participation of endogenous thiolic compounds in pain transmission through the administration of BSO, an inhibitor of glutathione synthesis. Twenty-four hours after treatment, we found a reduction in thiolic levels in the spinal cord paralleled by an increase in the sensitivity of mice to noxious (thermal and chemical hyperagesia) and innocuous (mechanical allodynia) paw stimulation. These findings demonstrate the participation of endogenous thiolic compounds on pain regulation.

Upon revealing the importance of both exogenous and endogenous thiolic compounds in an experimental pain model, we next tested the role of thiolic compounds in pain models that are more clinically relevant. We used 2 different models of inflammatory pain, a model of persistent pain (CFA-induced arthritis) and a model of acute pain (postincisional pain). In CFA-induced arthritis, NAC produced an antinociceptive effect with a longer time course than in the capsaicin test for both intraperitoneal and intrathecal administration. We also observed a greater reduction in nonprotein thiol levels in the spinal cord than in the capsaicin test. However, in the postincisional pain model, NAC showed no antinociceptve effect or alteration in nonprotein thiol levels in the spinal cord. This finding may be explained by the lack of participation of NMDA receptor involvement in the spinal cord during postincisional pain.[24] In contrast, the importance of NMDA receptors in CFA-induced arthritis has previously been shown.[41]

Taken together, our results demonstrate the importance of both exogenous and endogenous spinal nonprotein thiolic compounds in pain transmission. NAC is already clinically used with safety, so it could be used as an adjuvant in the treatment of pain.

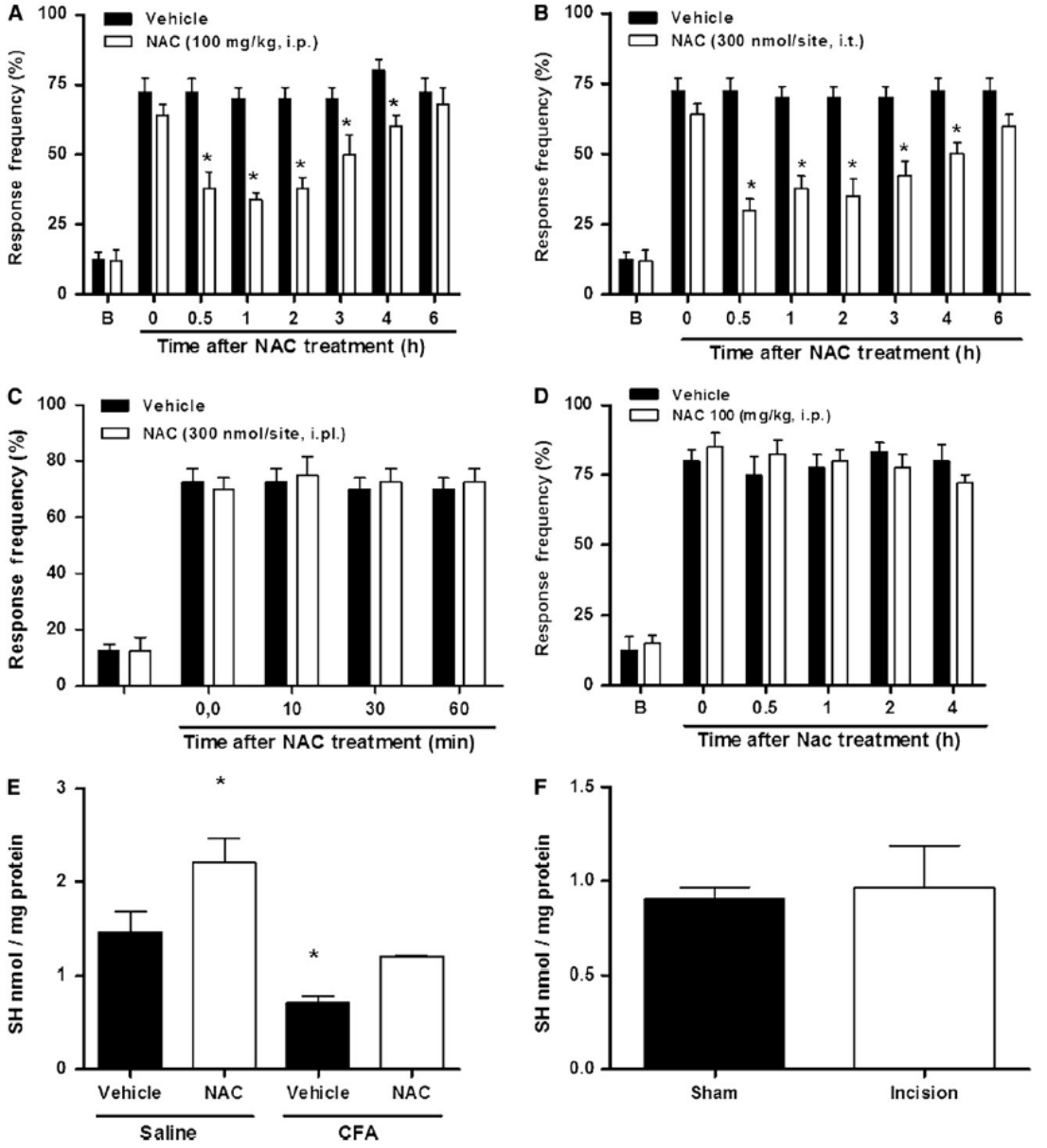

Figure 5. NAC (N-acetylcysteine) effect over alodynia elicted by von Frey Hair (VHF) stimulation in clinically relevant pain models. Anti-allodynic effect of NAC treatment (intraperitoneal **A**; intrathecal **B**) in the CFA-induced arthritis and lack of effect of NAC intraplantar **(C)** in the same model (n = 5). Lack of anti-alodynic effect of NAC intraperitoneal treatment in the surgery pain model **(D)**. Spinal levels of nonprotein thiol levels in the CFA-induced arthritis **(E)** and paw surgery model **(F)** (n = 4). Data are expressed as mean ± SEM. *$P < .05$, based on 1-way ANOVA **(E, F)** and 2-way ANOVA **(A, B, C, D)** followed by SNK post hoc test.

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4. MANUSCRITO

TRPV1 RECEPTOR ACTS AS A TARGET FOR NITRIC OXIDE DURING NOCICEPTION TRANSMISSION IN MICE SPINAL CORD

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ABSTRACT

Nociception may be modulated by reactive species (RS) production, including nitric oxide (NO) at spinal cord by yet poorly understanding molecular mechanisms. Since NO may activate TRPV1 receptor *in vitro*, an important receptor for pain transmission at spinal cord, we decided to investigate if nociception change NO levels at spinal cord and it as activate the transient receptor potential vanilloid 1 (TRPV1). Firstly, we observed that both intraplantar capsaicin (CAP) and intratecal L-arginine (ARG, the NO synthase substrate) induced thermal hyperalgesia, decreased non-protein thiol (SH) and increased nitrite/nitrate (NOx) levels, a stable metabolites of NO. Intrathecal pre-treatment with either NAC (SH donor) or L-NAME (NO synthase inhibitor) prevented hyperalgesia, decreased in SH and increased in NOx. Similarly, TRPV1 receptor antagonism, defunctionalization or knockdown reduced thermal hyperalgesia induced by intraplantar CAP and intrathecal ARG. Finally, we also observed the development of thermal hyperalgesia after intrathecal injection of endogenous SH synthesis inhibitor or with NO donors, all hyperalgesic behavior prevented by NAC and TRPV1 antagonism, while L-NAME only prevented the hyperalgesia induced by inhibition of SH synthesis. Thus, we may suggest that NO may induce nociception by spinal activation of TRPV1.

Key words: SIN1, L-arginine, nitroglycerine, capsaicin, biotin, BSO.

1. INTRODUCTION

The spinal cord plays a major role as integrative center of all peripheral nociceptive stimuli (Scholz J1, Woolf, 2002). Thus, the study of spinal mechanisms of pain transmission is relevant not only to understand it, but also to identify new targets and pathways to better control painful diseases. Of note, it has been described that free radicals (FR's) non protein thiol content (SH) may exert a critical role in the nociception transmission at spinal cord level (Rossato et al., 2010).

FR's are molecules that present unpaired electrons; which confers instability and elevated reactivity, which elicit they to react nonspecifically with different targets (Halliwell and Gutteridge, 2000). During normal metabolism, FR's are produced mainly by mitochondria, but also by several enzymes, as nitric oxide synthase (NOS), which oxidases L-arginine to L-citrulline to release nitric oxide (NO) (Luo and Cizkova, 2000). To avoid this nonspecific reaction, there are several systems to neutralize FR's. The most important among these are the SH compounds, represented mainly by free cysteine and glutathione (Martínez-Ruiz et al., 2011). Recently, it was demonstrated that the thiol antioxidants as N-acetylcysteine (NAC) induces antinociception in animal models and humans bearing neuropathy from different etiologies, which seems to be related to its antioxidant effect at spinal cord (Perez et al., 2003; Naik et al., 2006; Wolf et al., 2008; Rossato et al., 2010). Despite it, the real mechanism by which FR and SH modulate nociception is still unknown.

Therefore, it was already demonstrated that NO (a nitrogen FR) may induce nociception and, recently, that it may activate the transient potential

receptor vanilloid 1 (TRPV1) *in vitro* (Masue et al., 1999; Sousa and Prado, 2001; Schlechtweg et al., 2009; Miyamoto et al., 2009).

This receptor is a member of the transient potential receptor, formed by six transmembrane domains, with a pore permeable by cations, mainly calcium. It may be activated by several stimuli, as acidification, noxious heat and e TRPV1 participation in nociception was already demonstrated in several models of pain not only at peripheral level, but also at spinal cord (Spicarova et al., 2014).

Thus, we aim to investigate the relation between NO and SH levels changes during nociception. Also, we pretend to explore the correlation of these changes whit the TRPV1 activation at spinal cord.

2. MATERIALS AND METHODS

2.1. Animals

Male albino Swiss mice (20-30 g) bred in-house were used in all experiments. Animals were kept in a controlled environment (22±2°C) with a 12 hours light/dark cycle and fed standard lab chow and tap water *ad libitum*. Before the experiments, the animals were acclimatized to the laboratory for at least 1 hour and were used only once in each test. The experimental protocols were authorized by Ethics Committee of the Federal University of Santa Maria (CEUA, process number 124/2011) and are in accordance with current ethical guidelines for the investigation of experimental pain in conscious animals (Zimmermann, 1983). In addition, the number of animals and intensity of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments.

2.2. Reagents

The following reagents were purchased from Sigma: Butionine-sulfoxamine (BSO), capsaicin, N-acetylcysteine (NAC), N_{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), SB366791, sulfanilamide, N-(1-Naphthyl) ethylenediamine dihydrochloride (NEED), vanadium chloride, 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), ethylenediamine tetra acetic acid (EDTA), phenylmethanesulphonyl fluoride (PMSF), sodium dodecyl sulphate (SDS), aprotinin, β-glycerolphosphate, sodium orthovanadate, 3-morpholinosydnonimine hydrochloride (SIN1), L-arginine (ARG), streptavidin−agarose, S-methyl methanethiosulfonate (MMTS). Monoclonal

anti-TRPV1 was purchased from Santa Cruz Biothecnology, biotin-HPDE were purchased from Thermo Scientific, nitroglycerine (NTG) was purchased from Cristalia®.

2.3. Drug treatment

To investigate the nociceptive/antinociceptive effects of different drugs used, we performed the free-hand intrathecal injection in non-anesthetized animals described by Hylden and Wilcox, (1980). Briefly, the intervertebral space between L5 and L6 was punctured directly using a 28-gauge needle attached to a Hamilton micro syringe. A total volume of 5 µL was administered intrathecally. A tail flick was used as an indication that the needle had penetrated the *dura*.

2.4. Thermal hyperalgesia measurement

To investigate the nociceptive changes induced by different treatments, we evaluated animal thermal sensitivity. Briefly, mice were held and the right hind paw was immersed in a water bath at 48°C. The time spent (latency) to remove the paw from the water was timed and considered as thermal sensitivity (in seconds). A decrease in the latency to remove the paw from the water after treatment was considered as thermal hypersensitivity (Womer et al., 1997). We choose to evaluate this nociceptive behavior because it is classically associated to TRPV1. To avoid tissue damage, animals were no exposed to the water bath for longer period than 20 seconds.

2.5. Algogenic-induced thermal hyperalgesia

To investigate the spinal participation of NO and TRV1 in nociception, we induced thermal hyperalgesia in mice, by intraplantar capsaicin (CAP – 3nmol/paw), intrathecal L-arginine (ARG – 10 nmol/site, i.t.), nitroglycerine (NTG – 50 nmol/site, i.t.) and SIN1 (40 nmol/site, i.t.) administration. Briefly, basal latency was measured, then mice received the different algogenic treatment and new measurements were made 15, 30, 45, 60 and 90 minutes after injection (Meller et al., 1992; Masue et al., 1999; Sousa et al., 2001,).

2.6. Spinal thiol depletion-induced thermal hyperalgesia

We already described that spinal thiol depletion induces an oxidative stress state followed by thermal hyperalgesia. Briefly, we firstly measured mice basal latency and injected BSO (3.2 nmol/site) intrathecally. Twenty four hours after, we performed a new latency measurement to confirm the development of thermal hyperalgesia (Rossato et al., 2010).

2.7. Investigation of spinal SH and NO in the thermal hyperalgesia induced by different algogen agents

To investigate the participation of spinal SH and NO in nociceptive transmission, we evaluate the anti-hyperalgesic effect of N-acetylcysteine (NAC – antioxidant, SH donor) and L-NAME (NO synthesis inhibitor) in the different algogen cited previously. Briefly, we treated mice intrathecally with NAC (3 – 300 nmol) or L-NAME (3 – 300 nmol) 15 minutes prior algogen injection. To evaluate NAC and L-NAME anti-hyperalgesic effect, we measured thermal latency (thermal hyperalgesia), 15 to 90 minutes after algogen injection.

2.8. Thiol measurement

After the algogenic tests, animals were sacrificed and the lumbar portion of the spinal cord (L1 to L6) was collected. To estimate total and non-protein thiol contained in the sample, tissue was homogenized in 1.0 mL of 0.02 M EDTA and maintained on ice. The homogenate was centrifuged at 4,000 g for 15 minutes at 4°C. The supernatant was then divided into two aliquots of 300 µl (A and B). Aliquot A was used to determine total thiol content. Aliquot B was incubated whit 50 µl of 50% TCA at 4°C for 60 minutes. Following incubation, aliquot B was centrifuged at 12,000 g for 5 minutes at 4°C, and the supernatant was collected to determine the non-protein thiol content in the sample. To determine total thiol content, 200 µl of Tris/HCl (200 mM, pH 8.9) and 20 µl of DTNB (2.5 mM) were added to a 100 µl sample in a microplate and incubated at room temperature for 5 minutes (Sedlak and Lindsay, 1968). Protein thiol content was calculated by subtracting the values of non-protein thiols from total thiols. The colour of the solution resulting from the reaction was measured at 405 nm with a FisherBiotech Microkinetics Reader BT 2000. Cysteine was used as a standard to determine the thiol (SH) content in the samples.

2.9. NOx measurement

To investigate the role of NO during the nociceptive transmission, we quantified its stable metabolites nitrate and nitrite (NOx), according to Miranda et al., 2001, with minor modifications. Briefly, animals were sacrificed and the lumbar portion of the spinal cord was homogenized in 300 µL of PBS (50 mM, pH 7.4), deproteinized by adding 50 µL of acetonitrile and 50 µL of $ZnSO_4$ (200 mM) and centrifuged at 16000 x g during 20 minutes, 4°C. After, 75 µL

supernatant was added to 25 µL of Griess (sulfanilamide 2% and NEED 0.16%; 1:1), 25 µL vanadium chloride (dissolved in 5% HCl) and incubated for 60 minutes at 37°C. The color developed was evaluated at 540 nm. Nitrate and nitrite were used as standard and the values were corrected by the amount of protein in the samples (Bradford, 1976).

2.10. 3-nitrotyrosine measurement

3-Nitrotyrosine (3-NT) immunoreactivity is a marker of nitrosative stress and was determined as previously described by Joshi et al. (2006). Briefly, spinal cord samples were homogenized in phosphate buffered saline solution (PBS – ph 7.4, 50 mM) and centrifuged at 5000 x g, during 10 minutes at 4°C. After, 5 µL of supernatant sample (5 µg/mL), 5 µL of 12% SDS and 5 µL of modified Laemmli buffer containing 0.125 M Tris base pH 6.8%, 4% (v/v) SDS, and 20% (v/v) glycerol were incubated for 20 min at room temperature, and the membranes were developed as described above except a 1:2000 dilution of anti-3-NT polyclonal antibody was used. Blots were dried, scanned with Adobe Photoshop, and quantified with Scion Image (PC version of Macintosh compatible NIH image).The 3-NT blot had a faint background that was corrected in image analysis.

2.11. Investigation of spinal TRPV1 in the thermal hyperalgesia induced by different algogen agents

To investigate the participation of spinal TRPV1 in nociceptive transmission, we evaluate the anti-hyperalgesic effect of pharmacological blockade, defunctionalization and knockdown of TRPV1, as follow:

Pharmacological blockade: A basal value of thermal sensitivity was performed as described before and SB366791, a TRPV1 antagonist, was intrathecally inject (1 nmol/site). Fifteen minutes after, different algogen were injected and new measurements of thermal sensitivity were performed 30 minutes after (Dose based on pilot experiments – data not shown).

Defunctionalization: To defunctionalizate the spinal TRPV1 positive fibers, we used the intrathecal CAP-induced desensitization. Briefly, basal thermal sensitivity was measured; animals were anesthetized with inhalatory isoflurane and 5 µL of a 2% capsaicin solution was intrathecally injected. One week after, new measurements were performed to evaluate the thermal sensitivity (Scherrer et al., 2009).

Knockdown: To induce a decrease in TRPV1 protein expression at spinal cord, we adapted the oligonucleotide antisense protocol described by Christoph and co-workers (2007). Briefly, we evaluate the thermal sensitivity of mice an injected during 3 days (twice a day) 10 µg/5 µL intrathecally. After this period, new measurements of thermal hyperalgesia were made and spinal tissue samples were collected to confirm the TRPV1 knockdown by western blot analysis.

2.11. Statistical analysis

Results are expressed as mean ± S.E.M. Statistical analyses were performed using t-tests, one-way or two-way analysis of variance (ANOVA), followed by Dunnet test or Student-Newman-Keuls (SNK) posthoc tests, respectively, when necessary. The level of significance was set at $p < 0.05$. The percent of maximal inhibition was determined for each experimental

group using the following formula: % Inhibition = 100 - [(Experiment x 100)/Control].

3. RESULTS

3.1. The antinociceptive effect of NAC and L-NAME in the CAP-induced hiperalgesia

Firstly, we decided to investigate the participation of spinal ROS and NO during peripheral nociception. For this, we treated animals intrathecally with NAC (100 nmol/site) or L-NAME (300 nmol/site) and after 15 minutes they were submitted to intraplantar CAP-induced hyperalgesia (3 nmol/site). NAC treatment induced antinociceptive (anti-hyperalgesic) effect 30 minutes after CAP injection, while L-NAME presented antinociceptive (anti-hyperalgesic effect) 30 and 45 minutes after CAP injection (Figures 1A and C). Thus, we selected the time-point of 30 minutes to investigate the effect of different doses of both NAC (3 – 100 nmol/site, i.t.) and L-NAME (10 – 300 nmol/site, i.t.). We observed that NAC presented and ED_{50} of 13.6 (9.4 - 19.7) nmol/site and E_{max} of 100.0 ± 9.5%; and L-NAME presented and ED_{50} 127.4 (56.3 - 288.3) nmol/site and E_{max} of 95.1±16.1% (Figures 1B and D).

3.2. The antinociceptive effect of NAC and L-NAME in the ARG-induced hiperalgesia

Once we hypothesized that NO may be the main reactive specie to induce nociception at spinal cord, we investigated the effect of NAC and L-NAME in the nociception (thermal hyperalgesia) induced by intratecal ARG (10 nmol/site, i.t.). Firstly, we administered NAC (100 nmol/site) and L-NAME (300 nmol/site), and 15 minutes later we injected ARG. We observed that both presented antinociceptive (anti-hyperalgesic) effect from 15 to 45 minutes after

ARG injection, with maximal effect after 30 minutes (Figure 2A and C). Thus, we selected the time-point of 30 minutes to investigate the effect of different doses of both NAC (3 – 100 nmol/site, i.t.) and L-NAME (30 – 300 nmol/site, i.t.). We observed that NAC presented and ED_{50} of 19.9 (13.5 - 29.6) nmol/site and E_{max} of 100.0 ± 12.1%; and L-NAME presented and ED_{50} 63.2 (33.0 - 121.0) nmol/site and E_{max} of 109.0±9.1% (Figures 2B and D).

3.3. The antinociceptive effect of NAC and L-NAME in the BSO, NTG and SIN1-induced hiperalgesia

Once we observed that NAC and L-NAME present anti-hyperalgesic effect in both CAP and ARG induced nociception, we decide to reinforce these results by investigating the nociception induced by BSO (glutathione synthesis inhibitor – inductor of oxidative stress) and the NO donors NTG and SIN1. NAC (100 nmol/site, i.t.) presented anti-hyperalgesic effect in all three models of thermal hyperalgesia, with E_{max} of 100.0 ± 12.1, 63.2 ± 10.5 and 90.0 ± 16.6%, respectively, while L-NAME inhibited only the hyperalgesia induced by BSO with an E_{max} of 58.8 ± 11.0% (Figure 3).

3.4. Biochemical changes induced by intraplantar CAP and intrathecal ARG-induced nociception

To confirm the importance of thiol compounds and NO at spinal cord, we measured it in lumbar spinal cord of mice treated with NAC (100 nmol/site) or L-NAME (300 nmol/site) 30 minutes after intraplantar CAP injection or intratecal ARG. It was possible to observe that CAP, at the same time that induced nociception, also induced a decrease in total non-protein thiol content, an

increase in the amount of NOx at lumbar spinal cord in 36.8 ± 8.8 and 83.4 ± 19.8%, respectively. Furthermore, the pre-treatment with NAC and L-NAME also prevented the decrease in SH levels (122.9 ± 42.1 and 47.9 ± 21.1%), the increase in NOx (104.4 ± 29.1 and 73.9 ± 7.1%) and 3-NT formation (71.4 ± 9.7 and 135.4 ± 6.9%, respectively) caused by intraplantar capsaicin (Figures 4A, B and E).

Similarly, intrathecal ARG injection also induced a decrease in the total non-protein thiol content and an increase in NOx in 57.9 ± 7.4 and 129.4 ± 25.7%, respectively. The pre-treatment with NAC and L-NAME also prevented the decrease in SH levels (58.0 ± 18.3 and 30.4 ± 8.3%) and the increase in NOx (100.9 ± 6.4 and 112.5 ± 18.9%) caused by intraplantar capsaicin (Figures 4C and D).

3.5. Anti-hyperalgesic effect of TRPV1 blockade, defunctionalization and knockdown in the thermal hyperalgesia induced by CAP and ARG

Once we observed that ROS and NO seem to induce nociception and that ROS scavenger and NO synthesis inhibition induces antinociception, we decide next to investigate if the TRPV1 participate of these processes. First, we treated two different groups of animals with SB366791 (1 nmol/i.t.) and 15 minutes later, submitted both to intraplantar CAP or intrathecal ARG and observed the development of thermal hyperalgesia. We observed that SB366791 fully prevented CAP and ARG-induced hyperalgesia in 84.0 ± 7.5 and 76.2 ± 7.5%, respectively (Figure 5A and B).

To reinforce these results, we decided to promote intrathecal TRPV1 desensitization, induce by capsaicin. One week after defunctionalization, we

injected intraplantar CAP and intrathecal ARG, and observed that animals treated with vehicle presented a latency time 62.4 ± 4.5% greater than non-desensitized animals. Plus, in desensitized animal, intraplantar CAP induced a hyperalgesia of 53.3 ± 4.0%, while in non-desensitized animals CAP induced a hyperalgesic effect of 30.6 ± 2.8%. Also, intrathecal ARG induced no hyperalgesic effect in desensitized animals (Figure 5C and D).

To finally confirm these results, we induced a transient ablation of TRPV1 by repeated intrathecal injection of TRPV1 antisense (AS). As observed in the desensitization protocol, animals treated with antisense presented a greater latency time than PBS or mismatch (MM) treated animals (72.2 ± 3.7%). Plus, intraplantar CAP induced a hyperalgesic effect of 50.0 ± 4.5% in PBS treated animals, 46.2 ± 3.5 in MM treated animals and 18.5 ± 4.0% in AS treated animals. Meanwhile, ARG induced hyperalgesia of 53.4 ± 3.6% in PBS treated animals and 46.6 ± 6.6% in MM treated animals, but no hyperalgesia in AS treated animals (Figures 5E and F).

3.6. Anti-hyperalgesic effect of SB366791 in the thermal hyperalgesia induced by BSO, NTG and SIN1

To investigate the possible effect of ROS and NO on the TRPV1, we investigated the effect of SB366791 in the nociception induced by BSO, NTG and SIN1. We observed that all treatments induced thermal hyperalgesia persistent, which was prevented by intrathecal SB366791 treatment in 88.2±17.1, 90.5±7.5, 94.7±21.4 and 100.0±2.2%, respectively (Figures 6A, B and C).

4. DISCUSSION

In this work we evaluated the hypothesis that nociception may elicit NO production at spinal cord and it activates the TRPV1. First, we observed that intraplantar CAP and intrathecal ARG induce thermal hyperalgesia, SH level reduction, NOx and 3-NT increase; all changes prevented by intrathecal NAC and L-NAME administration. Furthermore the pharmacological, functional and genetic ablation of TRPV1 also prevented CAP and ARG-induced thermal hyperalgesia. Likewise, SB366791 and NAC prevented the thermal hyperalgesia induced by the oxidative stress inductor BSO and the NO donor's nitroglycerine (NTG) and SIN1, while L-NAME prevented only the thermal hyperalgesia elicited by BSO. Thus, we conclude that RS contribute to nociception by NO production and consequent TRPV1 activation at spinal cord.

To start the investigation, we used the CAP-induced thermal hyperalgesia model, a classical nociceptive behavior associated to TRPV1 activation. This model (thermal hyperalgesia induced by subcutaneous capsaicin injection) is well used in both human and rodents to investigate nociceptive mechanism and antinociceptive effect (Pöyhiä and Vainio, 2006; Mohr et al., 2008). Also, as we hypothesized that NO production at spinal cord may be a critical event during nociception, we used the intrathecal ARG-induced thermal hyperalgesia, once ARG play as endogenous substrate for NO production trough NOS (Cury et al., 2011).

Thus, to first investigate the modulation exerted by SH compounds and NO in the nociception transmission at spinal cord, we investigated the possible antinociception (anti-hyperalgesic effect) induced by an antioxidant and a NOS

inhibitor. We choose the thiol compound NAC because it is already used clinically to treat several disturbances with safety. Also, we already characterized its antinociceptive and antioxidant effects in different nociceptive models (Viggiano et al., 2005; Hacimuftuoglu et al., 2006; Rossato et al., 2010; Li et al., 2007); and L-NAME, a non-selective NOS inhibitor, once we do not know which specific isoform may be responsible for NOS production at this site (spinal cord) (Finkel et al., 2012).

As expected, both treatments (NAC and L-NAME) prevented the thermal hyperalgesia induced by CAP and ARG. Despite it, we observed a different profile between CAP and AGR test: both NAC and L-NAME presented a longer and more intense effect in the ARG test than CAP. We believe that this discrepancy is due to the different mechanism between these tests. In the intraplantar CAP, the algogenic substance stimulate tot only the nociceptor, but also other non-neuronal cells in the tissue, creating a more complex event, while the intrathecal ARG trigger a much more focal stimuli, directly in the spinal cord.

These data are in accordance with previously works from our and other groups, showing the antinociceptive effect of antioxidants and NOS inhibitors in different pre-clinical models of nociception and humans bearing pain non responsive to classical analgesic drugs (Perez et al., 2003; Vaggiano et al., 2005; Lima-Júnior et al., 2007; Wolf et al., 2008; Rossato et al., 2010). In these cases, it was observed changes in oxidative stress markers, as decrease in SH levels and an increase in the NOx amount during nociception (Kundu et al., 2012; El Boghdady et al., 2012; Veselinovic et al., 2014). Similar results were observed in our nociceptive models: intraplantar CAP and intrathecal ARG-

induced thermal hyperalgesia were followed by a decrease in SH levels at spinal cord, as well as an increase in NOx and 3-NT. It was expected in the ARG test, as it act as a substract for NOS to produce NO, which is converted to NOx or may react with SH compounds. In addition, we have already described the intraplantar CAP injection is able to decrease the levels of SH spinal cord, but no change in the amount of NOx was described. Thus, it is probably like that this changes observed in SH levels may be explained by the increase in NO production (NOx), once thiol compounds are responsible for NO neutralization.

These ideas are reinforced by the fact that oxidant agents as BSO, NTG and SIN1 induces nociceptive changes (thermal hyperalgesia) by modulating the amount of SH, NOx and OONO at the site of injection. The nociceptive change induced by all these compounds was prevented by NAC treatment, while L-NAME prevented only the nociception induced by BSO. These results may be explained by the fact that L-NAME act primary by inhibiting NOS, and NTG and SIN1 act by donating NO and OONO independently of NOS activity. Thus, L-NAME can't prevent the increase in the amount of NO/OONO produced in these models, presenting no effect. Therefore, our results are in accordance with previous works showing that oxidative changes were indicated as inductors and/or maintainers of nociception (Furuta et al., 2012; Ungard et al., 2013). This fact is reinforced by some works showing that the administration of different RS's, as well as the induction of oxidative stress, leads to nociception or nociceptive changes, as allodynia and hyperalgesia (Yeo et al., 2008; Rossato et al., 2010; Trevisan et al., 2013).

Regardless the association between nociception and RS production, the mechanism involve in this relation was not yet elucidated. Despite it, some

possible targets have been pointed recently as sensors for endogenous oxidants. Among this, some receptors of the transient receptor potential channel (TRP) were implicated in this RS detection, mainly TRP vanilloid 1 (TRPV1) and ankyrin 1 (TRPA1) (Miyamoto et al., 2009; Nichio et al., 2013). Moreover, the application of NO donors in culture cell elicited calcium influx currents, which was prevented by TRPV1 and TRPA1 antagonists. Despite it, only the genetic ablation of TRPV1 channel prevented these currents (Miyamoto et al., 2009). Thus, as we observed an increase in NOx levels during nociception and NAC prevented it concomitantly with its antinociceptive effect, we hypothesized that NO produced at spinal cord could activate the TRPV1 channel to elicit/facilitate nociception transmission.

To explore this possibility, we utilized three different approaches: the pharmacological blockade of TRPV1 by the selective antagonist SB366791, the spinal defunctionalization of TRPV1 induced by high dose of the agonist capsaicin, or the genetic ablation of TRPV1 induced by the repeated intratecal antisense administration. Firstly, we observed that SB366791 fully prevented the thermal hyperalgesia elicited by CAP and ARG. This result clearly indicates the participation of spinal TRPV1 in both peripheral nociception and spinal NO-mediated nociception. After, we induced a spinal defunctionalization of TRPV1 with a high dose of capsaicin (2%). Seven days after this procedure, treated mice presented a decrease in thermal sensitivity (increase in the thermal latency). The same basal change was observed also for the animal treated repeatedly with antisense against TRPV1, but not with MM. This change was expected once TRPV1 is the main receptor responsible for the detection of

nocive heat (temperature > 42°C) and the protocol indeed induced a defunctionalization of TRPV1.

Both defunctionalization and genetic ablation of TRPV1 elicited a fully prevention of intrathecal ARG-induced thermal hyperalgesia, as observed for the SB366791 treatment, reinforcing the idea that NO indeed elicit nociception trough TRPV1 receptor. Despite it, defunctionalization and genetic ablation of TRPV1 prevented only partially the intraplantar CAP-induced thermal hyperalgesia. It may be explained by the fact that ARG play as a NO donor, a possible direct activator of TRPV1 in this context, and the direct activation of TRPV1 is the responsible by the thermal hyperalgesia observed. Meanwhile, in the CAP-induced thermal hyperalgesia occurs a direct activation of TRPV1 in the nociceptor, but also of non-neuronal cells present in the paw. All these different stimuli lead to a nociceptive input to spinal cord. At this site, the nociceptive input from the paw triggers several nociceptive pathways, all responsible for the thermal hyperalgesia observed (Latremoliere and Woolf, 2009).

The importance of spinal SH and NO are emphasized by the anti-hyperalgesic effect elicited by SB366971 on the thermal hyperalgesia elicited by BSO, NTG and SIN1.These results are in agreement with several pre-clinical studies (neuropathic, inflammatory and cancer pain/nociception) showing the importance of TRPV1 in different types of nociception by pharmacological blockade, defunctionalization and genetic ablation (Christoph et al., 2007; Hoffmeister et al., 2014).

This fact motivated the research for new molecules that could inhibit the TRPV1. Despite this importance for nociceptive at spinal cord, clinical trial using

different TRPV1 antagonists has failed due to the lack of nociceptive effect or induction of severe hyperthermia resistant to classical anti-hyperthermiants (Wong and Gavva, 2009). These results discouraged new researches for TRPV1 antagonists clinically. Therefore it is important to create new strategies to prevent the TRPV1 over activation than its direct blockade. In this view, our results indicate a new possible way to prevent the over activation of TRPV1 through the neutralizing the NO.

Moreover, antioxidant treatment already proves to be effective to decrease pain score in neuropathic patients, without elicit side effects (Edmonds et al., 1997; Perez et al., 2003; Santanam et al., 2013). It may be explained by the prevention of overt activation of TRPV1, but not by its full blockade. Thus, the use of antioxidants may be an important approach to achieve a proper analgesic/antinociceptive effect avoiding severe side effect.

5. FIGURES AND LEGENDS

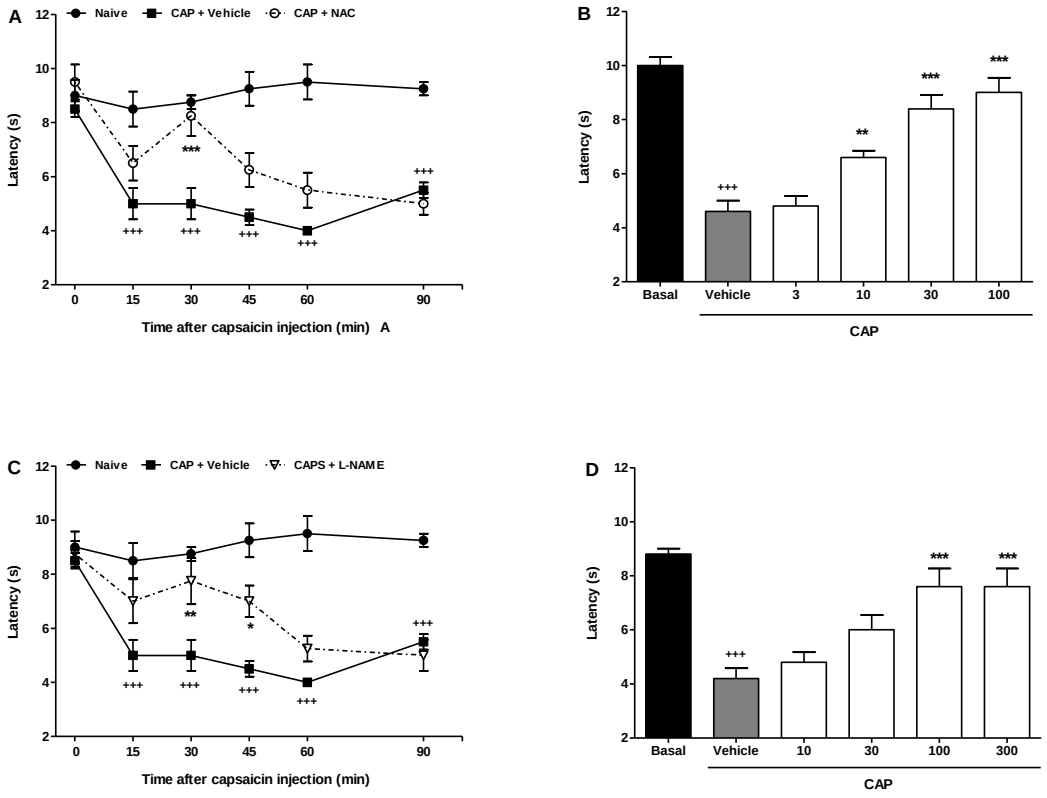

Figure 1: Antinociceptive effect of n-acetylcysteine and L-NAME on the thermal hyperalgesia induced by intraplantar capsaicin (CAP). (A) Time-course and (B) dose-response curves for the anti-hyperalgesic effect of NAC (3 – 300 nmol/site, i.t.); (C) time-course and (D) dose-response curves for the anti-hyperalgesic effect of L-NAME (3 – 30 nmol/site, i.t.) on the thermal hyperalgesia induced by CAP (3 nmol/paw). *p<0.05, *p<0.005 and ***p<0.001 in comparison the naïve or vehicle treated animals or basal; and $^{+++}$p<0.05 in comparison to CAP + vehicle treated animals, according to one or two way analysis of variance (ANOVA), followed by Bonferroni (A and C) or Dunnett (B and D) post-test (n = 5).

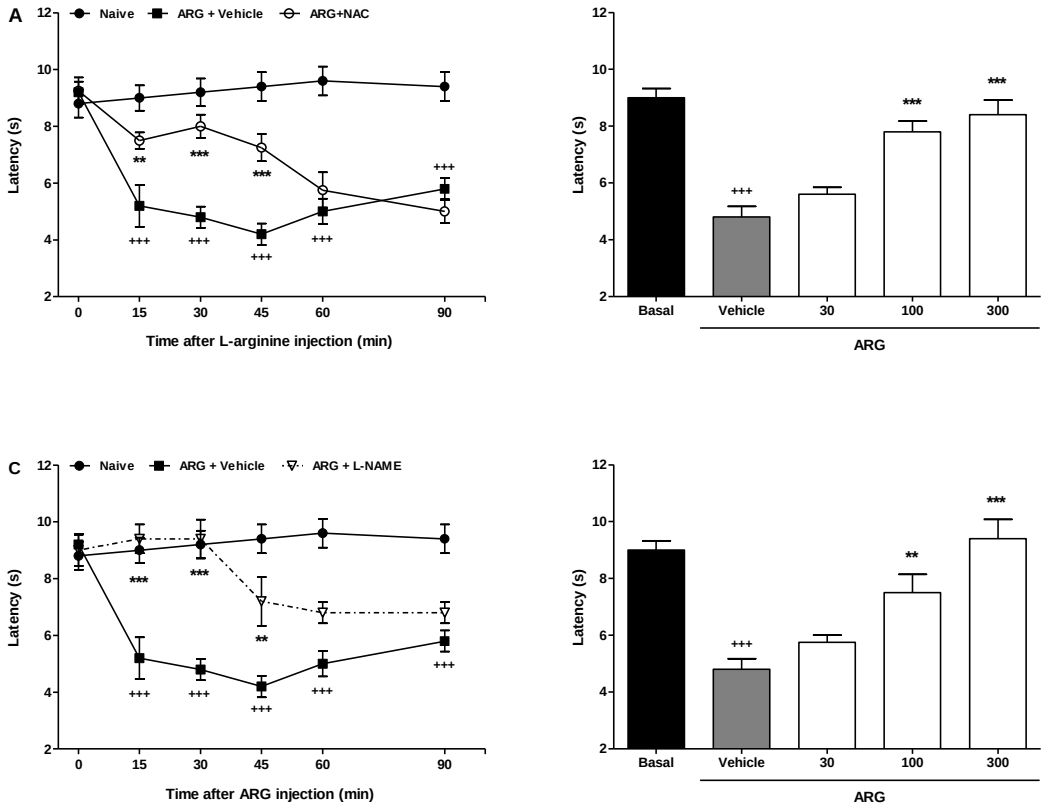

Figure 2: Antinociceptive effect of n-acetylcysteine and L-NAME on the thermal hyperalgesia induced by intrathecal L-arginine (ARG). (A) Time-course and (B) dose-response curves for the anti-hyperalgesic effect of NAC (3 – 300 nmol/site, i.t.); (C) time-course and (D) dose-response curves for the anti-hyperalgesic effect of L-NAME (3 – 30 nmol/site, i.t.) on the thermal hyperalgesia induced by ARG (10 nmol/paw). *p<0.05, **p<0.005 and ***p<0.001 in comparison the naïve or vehicle treated animals or basal; $^{+++}$p<0.001 in comparison to CAP + vehicle treated animals, according to one or two way analysis of variance (ANOVA), followed by Bonferroni (A and C) or Dunnett (B and D) post-test (n = 5).

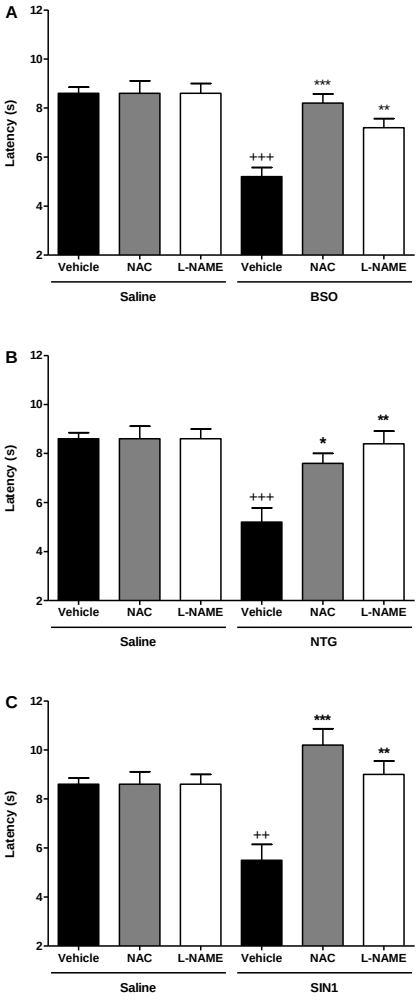

Figure 3: Antinociceptive effect of N-acetylcysteine (NAC) and L-NAME on the thermal hyperalgesia induced by non-protein thiol depletion and nitric oxide donors. Anti-hyperalgesic effect of NAC (100 nmol/site, i.t.) and L-NAME (300 nmol/ste, i.t.) on the thermal hyperalgesia induced by BSO (A - 3.2 nmol/site), NTG (B - 50 nmol/site, i.t.) or SIN1 (C - 40 nmol/site, i.t.). *p<0.05, **p<0.005 and ***p<0.001 in comparison the naïve or vehicle treated animals or basal; and $^{++}$p<0.005, $^{+++}$p<0.001 in comparison to nociceptive injection + vehicle treated animals, according to one way analysis of variance (ANOVA), followed by Dunnett post-test (n = 5).

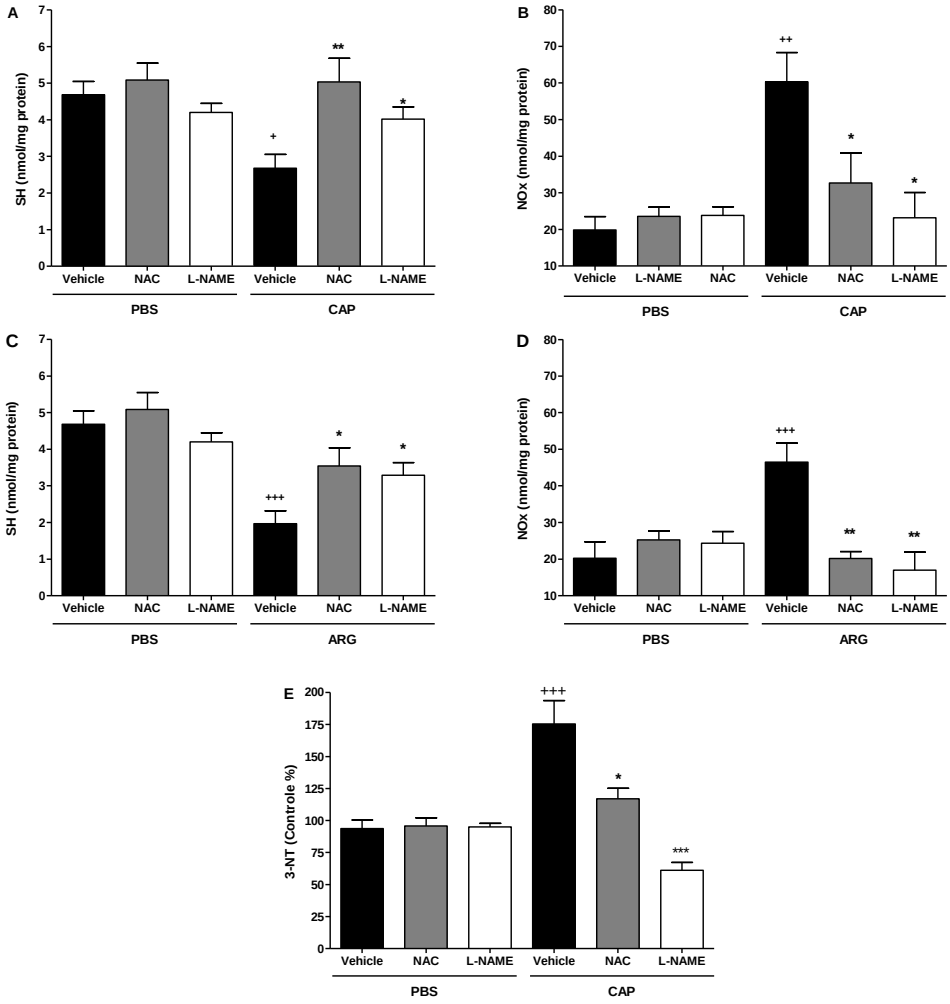

Figure 4: Effect of intrathecal N-acetylcysteine (NAC) and L-NAME on the biochemical changes induced by intraplantar capsaicin (CAP – 3 nmol/paw) and intrathecal L-arginine (ARG – 10 nmol/site, i.t.). Effect of intrathecal treatment with NAC and L-NAME on the decrease in non-protein thiol (A and C), increase in nitrite/nitrate (NOx - B and D) and 3-nitrotyrosine (3-NT – E) spinal levels after intraplantar CAP and ARG. *p<0.05, **p<0.005 and ***p<0.001 in comparison to vehicle-PBS treated animals; and $^{+}$p<0.05, $^{++}$p<0.005, $^{+++}$p<0.001 in comparison to nociceptive injection + vehicle treated animals, according to one way analysis of variance (ANOVA), followed by Dunnett post-test (n = 5).

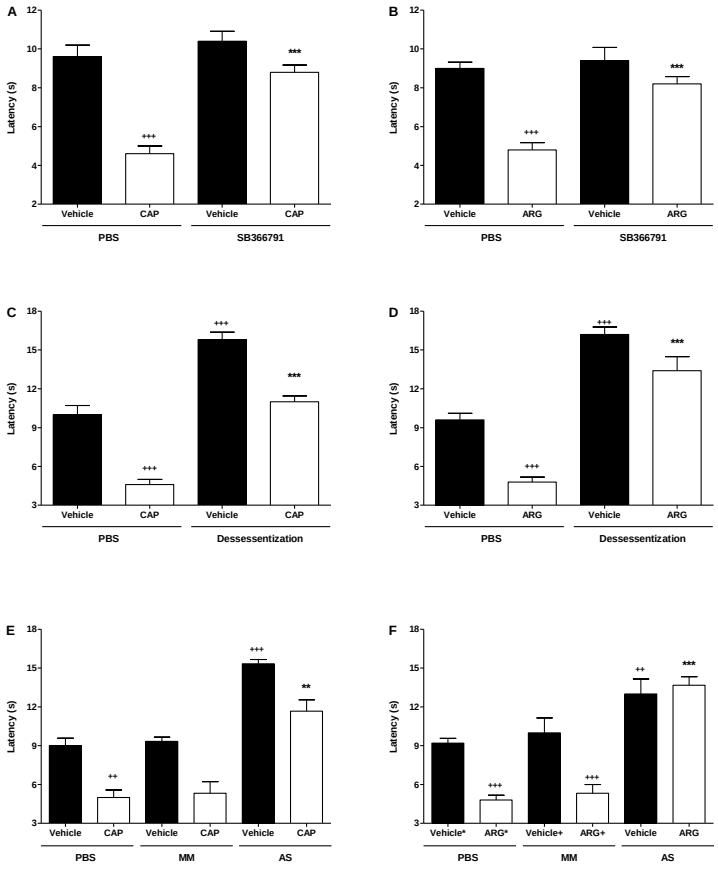

Figure 5: Participation of spinal TRPV1 in the thermal hyperalgesia induced by intraplantar capsaicin (CAP – 3 nmol/paw) and intrathecal L-arginine (ARG – 10 nmol/site, i.t.). Anti-hyperalgesic effect of the TRPV1 antagonist SB366791 (1 nmol/site, i.t. – A and B), intrathecal defunctionalization (C and D) and antisense-induced TRPV1 knockdown (E and F), but not the mismatch, on the thermal hyperalgesia induced by CAP and ARG. **p<0.005 and ***p<0.001 in comparison to vehicle-PBS treated animals, and $^{++}$p<0.005, $^{+++}$p<0.001 in comparison to nociceptive injection + vehicle treated animals, according to one way analysis of variance (ANOVA), followed by Dunnett post-test (n = 5).

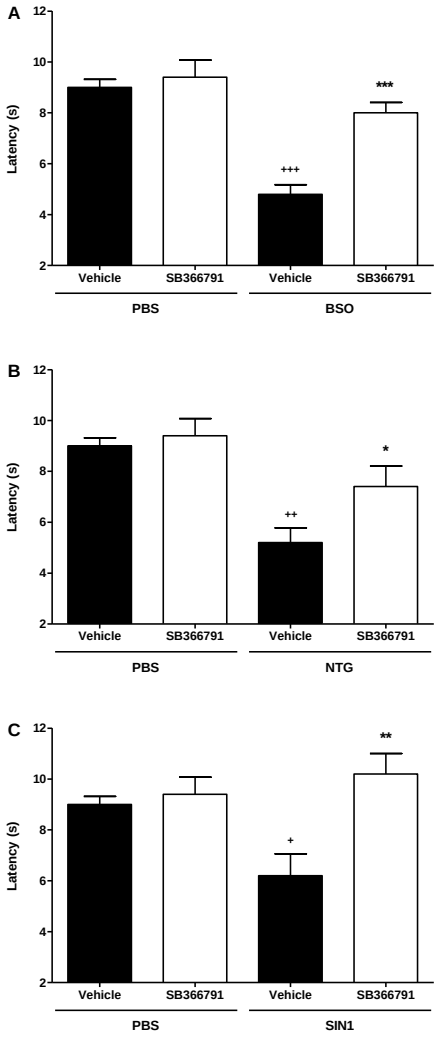

Figure 6: Effect of SB366791 (1 nmol/site, i.t.) on the thermal hyperalgesia induced by non-protein thiol depletion and nitric oxide donors. Anti-hyperalgesic effect of SB366791 on the thermal hyperalgesia induced by BSO (A – 3.2 nmol/site, i.t.), NTG (B – 50 nmol/site, i.t.) and SIN1 (C – 40 nmol/site, i.t.). *p<0.05, **p<0.005 and ***p<0.001 in comparison to vehicle-PBS treated animals; and $^{+}$p<0.05, $^{++}$p<0.005, $^{+++}$p<0.001 in comparison to nociceptive injection + vehicle treated animals, according to one way analysis of variance (ANOVA), followed by Dunnett post-test (n = 5).

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7. ARTGO 2

Artigo aceito para publicação, submetido à revista INFLAMMATION:

Anti-inflammatory effects of vitamin E on adjuvant-induced arthritis in rats

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ABSTRACT

Vitamin E (vit-E) is a lipophilic antioxidant, and its anti-inflammatory activity is still not full characterized. Thus, our goal was to investigate the anti-inflammatory effect of repeated vit-E treatment in the arthritis induced by the intraplantar injection of complete Freund's adjuvant (CFA). We observed an increase in arthritis scores, interleukin-1β and H_2O_2 levels, neutrophil and macrophage infiltration, thermal hyperalgesia, mechanical allodynia and loss of function induced by intraplantar CFA injection. These effects were unaltered after 1 day, partially reversed after 3 days and inhibited after 9 days after vit-E treatment. Furthermore, the concentration of Vit-E was reduced and that of tumor necrosis factor-alpha was increased in the CFA-injected paw. Both effects were reversed from 1 to 9 days after Vit-E treatment. However, Vit-E treatment did not alter CFA-induced edema at any time. Thus, Vit-E treatment produced an anti-inflammatory effect of slow onset in CFA, which demonstrates a disease-modifying drug profile.

Keywords: TNFα, IL1β, myeloperoxidase, NAGase, hydrogen peroxide.

1. INTRODUCTION

Vitamin E (vit-E) is the generic name for the phenolic compounds α, β, γ and Δ tocopherol, which consist of a chroman head and a phytyl tail. These compounds are lipophilic constituents of foods such as fish, seeds and cereals [1]. In mammals, vit-E is found in several compartments, including peripheral and central tissues, cerebrospinal fluid and plasma, occurring predominantly as α-tocopherol [2]. In cells, vit-E is the major lipid-soluble antioxidant, protecting membranes from free radicals [3]. This protection has been described as the main function of vit-E, and it confers beneficial effects against a large number of diseases, such as cardiovascular and neurodegenerative disorders [4, 5].

Furthermore, vit-E reportedly exerts an anti-inflammatory effect against several inflammatory diseases, such as hypersensitivity reactions, airway inflammation and several types of arthritis [6]. Patients with several types of arthritis exhibit reduced levels of vit-E in the plasma and synovial fluid [7, 8, and 9]. Also, it was demonstrated that adjuvant-induced arthritis increased serum markers of oxidative stress, some restored by vit-E chronic supplementation [10], while another study showed that prior vit-E deprivation exacerbate some inflammatory markers of collagen-induced arthritis [11]. Despite it, none evaluated no nociceptive/behavioral alteration, the most debilitant symptons or arthritis and the couses of patients to go for medical help.

However, clinical trials of vitamin E in the treatment of arthritis have yielded contradictory findings [12, 8]. Notably, a prospective placebo-controlled double-blind trial indicated that repeated Vit-E treatment reduced pain parameters but not clinical indices of inflammation in rheumatoid arthritis

patients [13]. In arthritis, a simple analgesic effect must be distinguished from an anti-inflammatory effect, as analgesics alleviate pain but do not stop disease progression [8]. Thus, our goal was to investigate the effect of repeated vit-E treatment on several nociceptive and inflammatory parameters in a rat model of arthritis induced by complete Freund's adjuvant (CFA).

2. MATERIALS AND METHODS

2.1. Animals

Male Wistar rats (250-350 g) bred in our animal housing facility were used. Animals were housed at a controlled temperature (22 ± 2°C) with a 12 h light/12 h dark cycle. Standard lab chow and tap water were available *ad libitum*. The animals were habituated to the experimental room for at least 1 h before experiments. Each animal was used only once, divided in four groups (saline i.pl. plus oil; saline i.pl. plus vit-E s.c.; CFA i.pl. plus oil s.c.; CFA i.pl. plus vit-E s.c.), 6 animals per group, in a total of four sets of experiments. Experiments were carried out in accordance with current ethical guidelines for the investigation of experimental pain in conscious animals [14] under process number CIETEA 124/2011 from the Council for Ethics in Animal Experimentation of UFSM.

2.2. Drugs and reagents

α-Tocopherol acetate was purchased from Delaware, Brazil, and diluted in mineral oil to the proper concentration. Complete Freund's adjuvant (CFA – 1 mg/mL suspension of heat-killed *Mycobacterium tuberculosis* in liquid paraffin), 5-(N,N-diethylamino)pentyl-3,4,5-trimethoxybenzoate (TMB), bovine serum albumin, Tween 20, hexadecyltrimethylammonium bromide (HTAB), p-nitrophenyl-2-acetamido-β-D-glucopyranoside (NAG) and horseradish peroxidase type II were purchased from Sigma. TNFα and IL-1β ELISA kits were purchased from R&D Systems. Butylated hydroxytoluene, phenol red and n-hexane were purchased from VETEC (Rio de Janeiro, Brazil).

2.3. CFA-induced arthritis

For the investigation of the anti-inflammatory and anti-nociceptive effects of vit-E, a CFA-induced arthritis model was used. Animals were lightly anesthetized with isoflurane, after which 100 µL of CFA or saline control was injected into the right hindpaw. After 72 hours, inflammatory and nociceptive parameters (arthritis scores – see below) were assessed to evaluate the progress of inflammatory processes.

2.4. Vit-E treatment

After the induction of inflammation, mineral oil (vehicle) or vit-E was administered in a single daily dose of 600 mg/kg. Twenty-four hours later, new measurements were made and another dose administered. Treatment was repeated for 9 days to evaluate the cumulative effect of vit-E. This dose was chosen because it has been shown to produce an antinociceptive effect when administered repeatedly in rats [15]. Given that vit-E was administer it by the subcutaneous route to avoid gastrointestinal irritation and gavage stress from repeated oral administration.

2.5. Arthritis score

To evaluate the progression of the arthritic response elicited by intraplantar CFA injection, animals were observed daily before vit-E administration. The following signs of inflammation were observed and classified according to the scale: edema formation (0 – normal; 1 – slight swelling at the injection site; 2 – swelling at the injection site and toes or ankle; 3 – swelling at the injection site, toes and ankle), redness (0 – normal; 1 –

slightly red/purple; 2 – red/purple) and claw position (0 – normal; 1 – slightly curved; 2 – almost closed). Individual scores were added to give the total arthritis score [16, 17].

2.6. Nociceptive evaluation

During inflammation, the release of chemical mediators leads to alterations in pain sensitivity, such as hyperalgesia (increased pain sensitivity) and allodynia (pain induced by a normally non-nociceptive stimulus) [18, 19]:

Thermal hyperalgesia: To evaluate hypersensitivity to heat stimulation, we used the Plantar Test apparatus (Ugo Basile, Varese, Italy) as described by Hargreaves and coworkers [20]. Briefly, animals were habituated to the place of observation, a Plexiglas chamber, for at least 30 minutes before the experiment. Afterwards, light from a 60 W bulb was directed at the right hind paw to determine the basal withdrawal latency. The time between stimulus onset and paw withdrawal was measured automatically, giving an index of the thermal nociceptive threshold. Significant decreases in paw withdrawal latency were interpreted as evidence of thermal hyperalgesia.

Mechanical allodynia: Mechanical allodynia was evaluated using the up-and-down method described by Dixon [21] with von Frey filaments. Briefly, animals were placed in cages with a wire mesh bottom permitting unobstructed access to the paws. A paw was touched with one of a series of 7 von Frey hairs in logarithmic increments (6, 8, 10, 15, 26, 60 and 100). The von Frey hairs were applied to the plantar surface at a perpendicular angle with sufficient force to cause slight buckling, then held for approximately 2–4 s. Stimuli were

presented at intervals of several seconds, allowing for the resolution of any behavioral responses to previous stimuli [22].

2.7. Assessment of loss of function

Because chronic inflammation compromises the function of the affected limb due to pain, edema and other alterations such as stiffness, we decided to investigate locomotor activity as a measure of hind-paw function. To evaluate this parameter, we used the open field test [23]. The apparatus was a circular open field (56 cm diameter) with the floor divided into 10 equal areas. Animals were observed in the open field for 5 minutes. The number of crossings was recorded as an index of locomotor activity. Locomotor activity was measured 1, 3 and 9 days after the start of vit-E treatment.

2.8. TNFα and IL-1β measurement

To investigate the involvement of tumor necrosis factor alpha (TNFα) and interleukin 1-beta (IL-1β) in the effects of vit-E, skin samples were collected from the injected paw and frozen until analysis. Samples were homogenized in 200 µL of phosphate-buffered saline (pH 7.4) containing 0.5% Tween 20, 0.1% bacitracin, 0.01 µg/mL soybean trypsin inhibitor, 0.5% bovine serum albumin and 10 mM EDTA [24]. The final homogenate was centrifuged at 16,000 g at 4 °C for 10 minutes and levels of TNFα and IL-1β in the supernatant measured using an ELISA kit.

2.9. Paw edema

To observe the development of edema, animals were held while right hind paw thickness was measured using a digital caliper [25]. Seventy-two hours after the induction of inflammation and before each dose of vit-E, new measurements were taken and compared to basal values. An increase in the difference between the measures was considered as representing edema Values are expressed as the change relative to basal values.

2.10. Cell infiltration markers

To estimate the effect of vit-E treatment on inflammatory cell infiltration, samples were collected to estimate the activity of N-acetyl-β-D-glucosaminidase (NAGase) and myeloperoxidase (MPO) [26, 27, respectively], which are markers of macrophage and neutrophil infiltration, respectively. One, three and nine days after treatment with vit-E, paw skin samples were collected and frozen until analysis. Samples were homogenized in acetate buffer (8 mM, pH 5.5) containing 0.5% HTAB and centrifuged at 16,000 g at 4 °C for 20 minutes. The supernatant was divided into two aliquots.

Macrophage infiltration was estimated by measurement of NAGase activity. Briefly, 25 µL of supernatant was incubated with 25 µL of *p*-nitrophenyl-2-acetamido-β-D-glucopyranoside (2.24 nM) and 100 µL of citrate buffer (50 mM, pH 4.5) at 37 °C for 1 hour. Afterwards, 100 µL of glycine buffer (0.2 µM, pH 10.4) was added to stop the reaction and to allow the development of color, which was read at 405 nm. To evaluate neutrophil infiltration, myeloperoxidase activity was also evaluated. Briefly, 10 µL of supernatant was added to 200 µL of acetate buffer and 20 µL of TMB (18.4 mM) and incubated at 37 °C for 3

minutes. To stop the reaction, microplates were placed on an ice bath and 30 µL of acetic acid added. The color formed was assessed at 630 nm.

Both reactions were read in a Fisher Biotech Microkinetics BT 2000 microplate reader. Values are expressed as optical densities corrected for protein content measured using the Coomassie dye method [28].

2.11. Hydrogen peroxide quantification

To determine the level of H_2O_2 in paw skin after CFA injection we used the phenol red-HRPO method [29]. Briefly, samples were homogenized at 4 °C in 300 µL of 50 mM phosphate buffer (pH 7.4) containing 5 mM sodium azide. The homogenate was then centrifuged at 12,000 g for 20 min at 4 °C. Supernatant (200 µL) was mixed with 75 µL of phenol red (100 µg/mL) and 15 µL of HRPO (50 µg/mL). The mixture was incubated for 10 minutes at 25 °C, and then the reaction was stopped by the addition of 20 µL NaOH (1 M). Absorbance was recorded at 610 nm using a Fisher Biotech Microkinetics BT 2000 (Fisher Scientific, Pittsburgh, PA, USA) microplate reader. H_2O_2 levels are expressed as nanomoles of H_2O_2 equivalent per skin sample, based on a standard curve of HRPO-mediated oxidation of phenol red by H_2O_2 corrected for protein content [28].

2.12. Vitamin E measurement

To determine if vit-E administration (600 mg/kg, s.c.) increased levels of vit-E in both serum or paw of treated animals, we used a modification of the method described elsewere [30]. Briefly, paw skin samples were homogenized in 300 µL of 66% ethanol and centrifuged at 16,000 g at 4 °C for 10 minutes.

Supernatant or serum samples were used to measure vitamin E levels. The reaction was prepared in a covered tube by mixing 140 µL of Milli-Q water, 20 µL of BHT (10 mM in 66% ethanol), 140 µL of sample and 2.1 mL of 66% ethanol. The mixture was then vortex-mixed for 10 s; 3.5 mL of n-hexane was added, and the mixture was mixed for another minute, followed by centrifugation at 2,000 g for 5 minutes. Then 1 mL of supernatant was transferred to a fluorimeter cuvette and the concentration of vitamin E was measured in the fluorimeter (excitation 295 nm, emission 340 nm). Calibration curves with α-tocopherol were used to determine the concentration, following the same procedure as used for the experimental samples.

2.13. Statistical Analysis

Results are expressed as mean ± S.E.M. Data were analyzed by two-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test. The level of significance was set at $p < 0.05$. Vit-E effect was calculated as inhibition of the changes induced by CFA, using the following formula: Inhibition (%) = 100 - [(Experiment × 100)/Control].

3. RESULTS

3.1. Effect of Vit-E on CFA-induced inflammation

To characterize the overall effect of vit-E on chronic inflammation induced by CFA, we assessed arthritis scores. Intraplantar CFA, but not saline, induced a large increase in arthritis scores as early as 72 hours after injection (day 0 of vit-E or vehicle treatment) (Figure 1). After the establishment of inflammation, we started daily subcutaneous treatment with vit-E (600 mg/kg) or vehicle. Arthritis scores remained high in animals injected with CFA and repeatedly treated with vehicle (oil) from 1 to 9 days after treatment started. In CFA-injected rats, Vit-E reduced arthritis scores just 3 days after treatment started, an effect that gradually increased, reaching its maximal value (96±4% inhibition) at day nine (Figure 1). No considerable physical change was observed during the treatment, as skin irritation/inflammation in the points of administration or body wheit.

Thus, the next measures were carried out at three different times after treatment started: day 1 (when vit-E did not produce any effect on arthritis scores), day 3 (when vit-E produced a partial effect) and day 9 (when vit-E produced a full effect).

3.2. Effects of CFA and vit-E treatment on total vit-E and hydrogen peroxide (H_2O_2) content in paw skin

We next evaluated levels of vit-E in paw skin during treatment. CFA induced a decrease in total vit-E levels compared to saline one, three and nine days after vehicle (oil) treatment started (Figure 2A). Vit-E fully restored vit-E

levels in CFA-injected animals at days three and nine after treatment started. Vit-E treatment did not alter paw skin vit-E levels in rats injected with saline (Figure 2A). Additionally, CFA did not change total vit-E serum levels during the period of analysis, but levels were increased by vit-E after three and nine days of treatment (data not shown).

Because vit-E is an antioxidant, we also investigated its effect on levels of the reactive oxygen species H_2O_2 in paw skin samples. CFA induced an increase in H_2O_2 levels compared to saline from days one to nine in vehicle-treated rats (Figure 2B). Vit-E treatment reduced this increase in H_2O_2 at day three (61±7%) and abolished it at day nine (100%) after treatment started in CFA-injected rats (Figure 2B). Vit-E treatment did not alter paw skin H_2O_2 levels in rats injected intraplantarly with saline (Figure 2B).

3.3. Vit-E effect in nociception and loss of function induced by CFA

One of the major debilitating effects of arthritis is the development of painful hypersensitivity (hyperalgesia and allodynia) and loss of function in inflamed tissue (assessed here as a decreased number of crossings in an open field). Compared to saline-injected animals, CFA produced marked decreases in thermal withdrawal latency (thermal hyperalgesia), mechanical threshold (mechanical allodynia) and locomotor function (loss of function) in vehicle (oil)-treated rats (Figures 3A-C). No beneficial effect was observed after one day of vit-E treatment in CFA-injected animals. After three days of treatment, vit-E (600 mg/kg, s.c.) reduced hyperalgesia, allodynia and loss of function by 29±4, 21±8 and 95±9%, respectively, in CFA-injected animals. The inhibitory effects of vit-E increased by the ninth day, when we observed 100% inhibition of CFA-induced

hyperalgesia, allodynia and loss of function (Figures 3A, B and C). On the other hand, vit-E treatment did not alter thermal latency, mechanical threshold or number of crossings in rats injected intraplantarly with saline (Figure 3A-C).

3.4. Cytokine production

Next, we evaluated the effect of vit-E treatment on the production of pro-inflammatory cytokines TNFα and IL-1β. Compared with saline-injected rats, tissue TNFα and IL-1β levels increase in CFA-injected animals from one to nine days after vehicle treatment started (Figures 4A and B). Vit-E treatment abolished the increase in TNFα from day one until day nine, while IL-1β only returned to normal levels three to nine days after vit-E treatment started in CFA-injected rats (Figure 4A and B). However, vit-E treatment did not alter TNFα or IL-1β levels in rats injected intraplantarly with saline (Figure 4A and B).

3.5. Vit-E effect on CFA-induced edema and leukocyte infiltration

We observed an increase in paw thickness (edema) in all animals injected with CFA, but not in saline-injected rats treated with vehicle (Figure 5A). However, vit-E treatment failed to reduce paw edema caused by CFA during the period analyzed (Figure 5A).

We observed an increase in both NAGase and MPO activity (indicators of neutrophil and macrophage infiltration, respectively) in CFA-treated animals compared to saline after vehicle treatment (Figures 5B and C). Rats injected with CFA and treated with vit-E showed a decrease in MPO activity from the third day after the commencement of vit-E treatment (45±11%) which lasted until the ninth day, when MPO activity returned to basal levels (Figure 5B).

NAGase returned to basal levels at day three; this effect lasted until the end of the treatment (Figure 5C). Vitamin E treatment did not induce any alteration of NAG and MPO activity in saline-injected animals (Figures 5B and C).

4. DISCUSSION

Vit-E is a lipid-soluble vitamin found in several foods with outstanding antioxidant activity. Analgesic and anti-inflammatory activities of vit-E have been reported in both humans and rodents [31] for hypersensitivity reactions, airway inflammation diseases and several types of arthritis, but contradictory findings have also been reported [6]. Here, we show that vit-E treatment reduced arthritis scores and nociceptive and inflammatory parameters (hyperalgesia, allodynia, loss of function, cellular infiltration and cytokine production) but did not inhibit edema formation.

To investigate the anti-inflammatory effect of vit-E, we tested it against inflammation induced by CFA, an animal model of chronic inflammation with some similarities to forms of arthritis such as rheumatoid and osteoarthritis [31]. First, we evaluated overall arthritic state using an arthritis score index that included important measures such as mobility and erythema, which are also influenced in human arthritis [32]. After a daily dose of 600 mg/kg, we observed slow-onset effect, which commenced only three days after the beginning of the treatment, progressing to full recovery at day nine. Using this arthritic score profile, we selected three time points to investigate other features of inflammation: day one, when there is no effect on arthritis score; day three, when the effect commences; and day nine, the time of maximal effect.

The slow onset of vit-E's effects may be caused by slow distribution of vit-E in the inflamed tissue). In fact, treatment induced an increase in serum levels only at days three and nine: no effect was observed at day one (data not shown). At the same time, CFA induced a decrease in paw skin vit-E levels in

vehicle-treated animals. These results indicate that vit-E may be more effective at the site of inflammation than sistemicaly. Similar results have been reported in humans. Some studies have shown a significant decrease in vit-E levels in the synovial fluid of patients with active osteo/rheumatoid arthritis [8, 33]. Vit-E supplementation restored these levels while simultaneously inducing an antinociceptive effect, in both humans and rodents [8,33]. The reasons for vitE's importance at the site of inflammation are not fully understood but may be related to its antioxidant effects.

It is known that inflammatory processes induce an increase in the production of reactive species such as hydrogen peroxide (H_2O_2) [34]. These reactive species are produced by MPO, NDPH oxidase and other sources. It is now known that H_2O_2 modulates inflammatory responses, altering the function of several proteins and activating receptors (as TRPA1 and TRPV1) and enzymes (protein kinase C – PKC) [35, 36, 37]. All these targets are involved in triggering inflammatory processes, nociception and cytokine production/release. In this study, CFA induced an increased production of H_2O_2, which was inhibited by vit-E treatment from day three to nine. This outcome was expected, as vit-E possesses marked antioxidant properties and can neutralize various reactive species. It was also already known that vit-E prevents changes induced by H_2O_2 including DNA damage, lipid peroxidation and changes in protein expression [38]. Thus, it is possible that vit-E may act by neutralizing H_2O_2 and thus preventing the inflammatory changes observed after CFA injection or in arthritis.

Clinically, the most debilitating effects of inflammatory processes are sensory alterations, such as pain during movement or stimulation. This pain is the main motivation in patients seeking medical care [8]. In this study, we

observed that CFA induced marked mechanical allodynia and thermal hyperalgesia: sensory alterations induced by inflammation in both humans and rodents [19, 18]. No immediate acute effect of treatment was observed. Antinociception was only apparent after 3 days of treatment, reaching a maximum at day nine. Similarly, mechanical allodynia induced by CFA was also prevented from days three to nine. This time-course correlates with the restoration of vit-E levels at the site of inflammation. Antinociception induced by vit-E was also described by Kim and co-workers in an animal model of neuropathy: vit-E (100 mg/kg, i.p.) treatment induced an anti-allodynic effect after 3 days of treatment [15].

Due to inflammatory processes, arthritis commonly promotes loss of function in the affected limb [39]. We decided to investigate the spontaneous locomotion of animals in the open field test after intraplantar injection of CFA. As expected, CFA decreased the number of crossings in the open field. Vit-E inhibited this effect. These results are in agreement with data from Edmonds and co-workers [13] on the analgesic effect of chronic vit-E treatment in humans. In that work it was shown that vit-E decreased pain in various situations, such as in the morning, during movement and after rest. Beyond a simple analgesic effect, vit-E also reversed debilitating symptoms elicited by painful inflammation [13]. Vit-E treatment did not alter any motor or sensory measure in animals treated with intraplantar saline.

Despite the complex mechanisms of inflammation, several substances produced at the site of injury play a role in producing and sustaining nociception. Of these substances, cytokines play a major role [40]. It was previously reported that one of the first changes in CFA-induced inflammation is

the production of TNFα. This cytokine contributes to the development of nociceptive changes such as thermal hyperalgesia, as well as to the increase in the levels of other pro-inflammatory cytokines such as IL-1β [40]. In the same manner, IL-1β also acts as a hyperalgesic cytokine, inducing the release of other mediators [39]. Therefore, we investigated the effect of vit-E treatment on CFA-induced cytokine production. We observed increases in TNFα and IL-1β levels during the period of observation. Vit-E prevented these increases from days three to nine for TNFα, and from days one to nine for IL-1β. These inhibitory effects on cytokine release or production have also been reported in humans with arthritis and in several animal models of inflammation [41, 42, 43]. Nonetheless, we did not investigate the mechanisms involved in the liberation of these cytokines; the temporal profiles observed indicate that the initial inhibition of IL-1β may be involved in other effects of vit-E.

In addition to the nociceptive effects of these cytokines, they also play important roles in chemotaxy and cell activation [40]. Once they infiltrate the site of inflammation, these cytokines produce a series of inflammatory mediators to remodel the injured tissue and neutralize the source of inflammation. During arthritic processes, the activity of neutrophils (infiltrated cells) and macrophages (differentiated monocytes) are very important to both processes [44]. Vit-E also prevented the increase in MPO and NAGase activity in paw skin: markers of neutrophil and macrophage cell infiltration, respectively. Despite this finding, no effect on edema formation was observed. This lack of effect may be due not only to the severe response elicited by CFA but also to the dose and time of treatment.

Taken together, our results indicate that vit-E may act as an anti-inflammatory treatment, targeting several points of the inflammatory process such as nociception, cytokine production and cell infiltration. Vit-E's antioxidant properties are presumably also important for its anti-inflammatory effect.

CONCLUSION

These experiments confirm the anti-inflammatory effect of repeated vit-E administration in a model of inflammation exhibiting several similarities with arthritis in humans. These data support clinical findings on the efficacy of vit-E in the treatment of several types of arthritis, indicating that this effect is related to its antioxidant effect.

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FIGURE LEGENDS

Figure 1: Effect of vit-E (600 mg/kg; s.c.) on CFA-induced increase in arthritis score. CFA, but not saline, induced an increase in arthritis score 72 h after CFA injection. This effect lasted from days one to nine, when we terminated observations. Vit-E decreased this score from days three to nine. The results are shown as median and interquartile range; $^{###}p<0.001$ in comparison to saline – oil group; $^{**}p<0.005$ and $^{**}p<0.001$ in comparison to CFA-oil group, based on two-way analysis of variance (ANOVA) followed by Bonferroni post-test (n = 6).

Figure 2: Changes in vit-E levels and hydrogen peroxide (H_2O_2) production induced by CFA and vit-E treatment (600 mg/kg; s.c.). Total (A) vit-E level and (B) H_2O_2 in paw skin one, three and nine days after treatment. The results are shown as mean ± SEM; $^{#}p<0.05$, $^{##}p<0.005$ and $^{###}p<0.001$ in comparison to saline – oil group; $^{*}p<0.05$, $^{**}p<0.005$ and $p<0.001$ in comparison to CFA-oil group, based on two-way analysis of variance (ANOVA) followed by Bonferroni post-test (n = 4).

Figure 3: Effect of Vit-E on nociceptive changes induced by CFA. (A) Thermal hyperalgesia, (B) mechanical allodynia and (C) loss of function induced by CFA, along with the effect of vit-E on days one, three and nine. The results are shown as mean ± SEM; $^{#}p<0.05$ $^{###}p<0.001$ in comparison to saline – oil group; $^{*}p<0.05$, $^{**}p<0.005$ and $^{***}p<0.001$ in comparison to CFA-oil group, based on

two-way analysis of variance (ANOVA) followed by Bonferroni post-test (n = 4-6).

Figure 4: Cytokine production in paw skin induced by CFA. (A) TNFα and (B) IL-1β levels on days one, three and nine after CFA injection and vit-E treatment. The results are shown as mean ± SEM; $^{\#\#}p<0.005$ and $^{\#\#\#}p<0.001$ in comparison to saline – oil group; $^{*}p<0.05$ and $^{**}p<0.005$ in comparison to CFA-oil group, based on two-way analysis of variance (ANOVA) followed by Bonferroni post-test (n = 6).

Figure 5: Effect of Vit-E on inflammatory changes induced by CFA. (A) Paw edema, (B) neutrophil infiltration (measured by MPO activity) and (C) macrophage activation (measured by NAGase activity) induced by CFA, along with the effect of vit-E on days one, three and nine. The results are shown as mean ± SEM; $^{\#}p<0.05$, $^{\#\#}p<0.005$ and $^{\#\#\#}p<0.001$ in comparison to saline – oil group; $^{*}p<0.05$, $^{**}p<0.005$ and $^{***}p<0.001$ in comparison to CFA-oil group, based on two-way analysis of variance (ANOVA) followed by Bonferroni post-test (n = 6).

Figure 1:

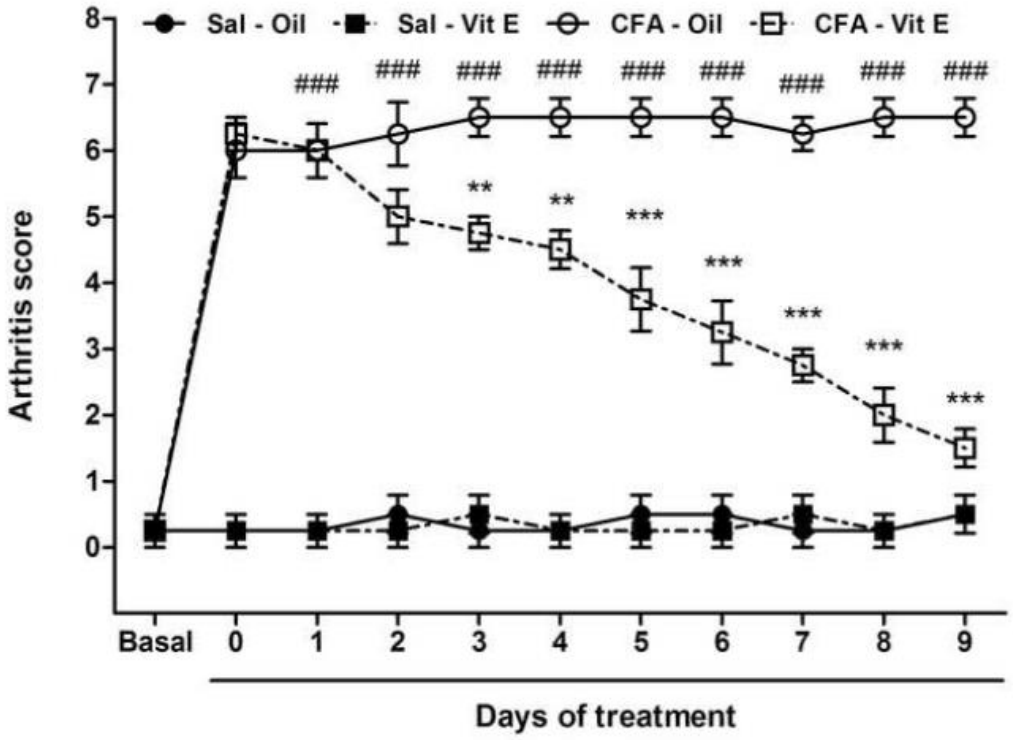
Sal - Oil
Sal - Vit E
CFA - Oil
CFA - Vit E
Arthritis score
Days of treatment
Basal
0
1
2
3
4
5
6
7
8
9
###
**
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Figure 2:

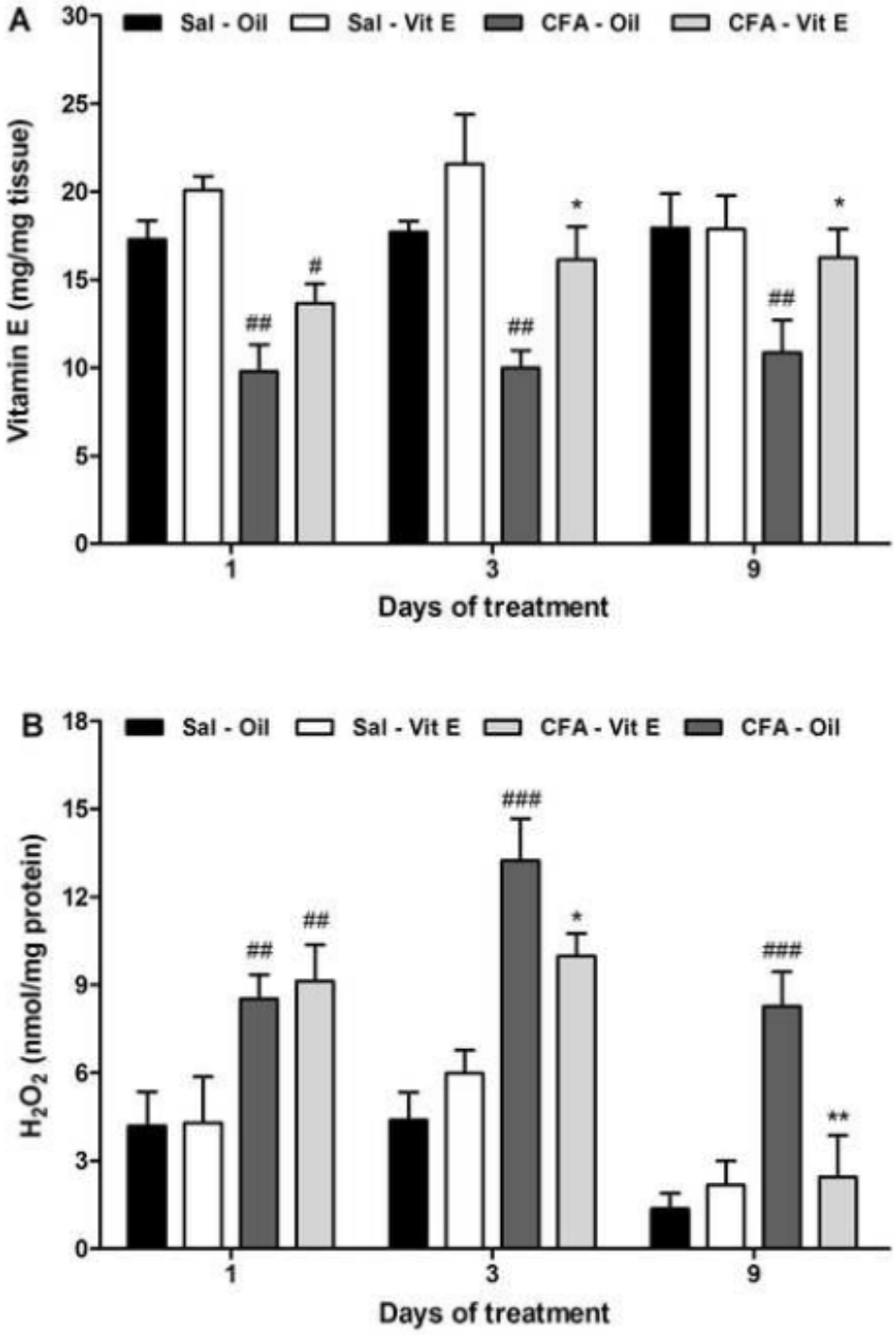

A
Sal - Oil
Sal - Vit E
CFA - Oil
CFA - Vit E
Vitamin E (mg/mg tissue)
30
25
20
15
10
5
0
##
#
##
*
##
*
1
3
9
Days of treatment
B
Sal - Oil
Sal - Vit E
CFA - Vit E
CFA - Oil
H2O2 (nmol/mg protein)
18
15
12
9
6
3
0
##
##
###
*
###
**
1
3
9
Days of treatment

Figure 3:

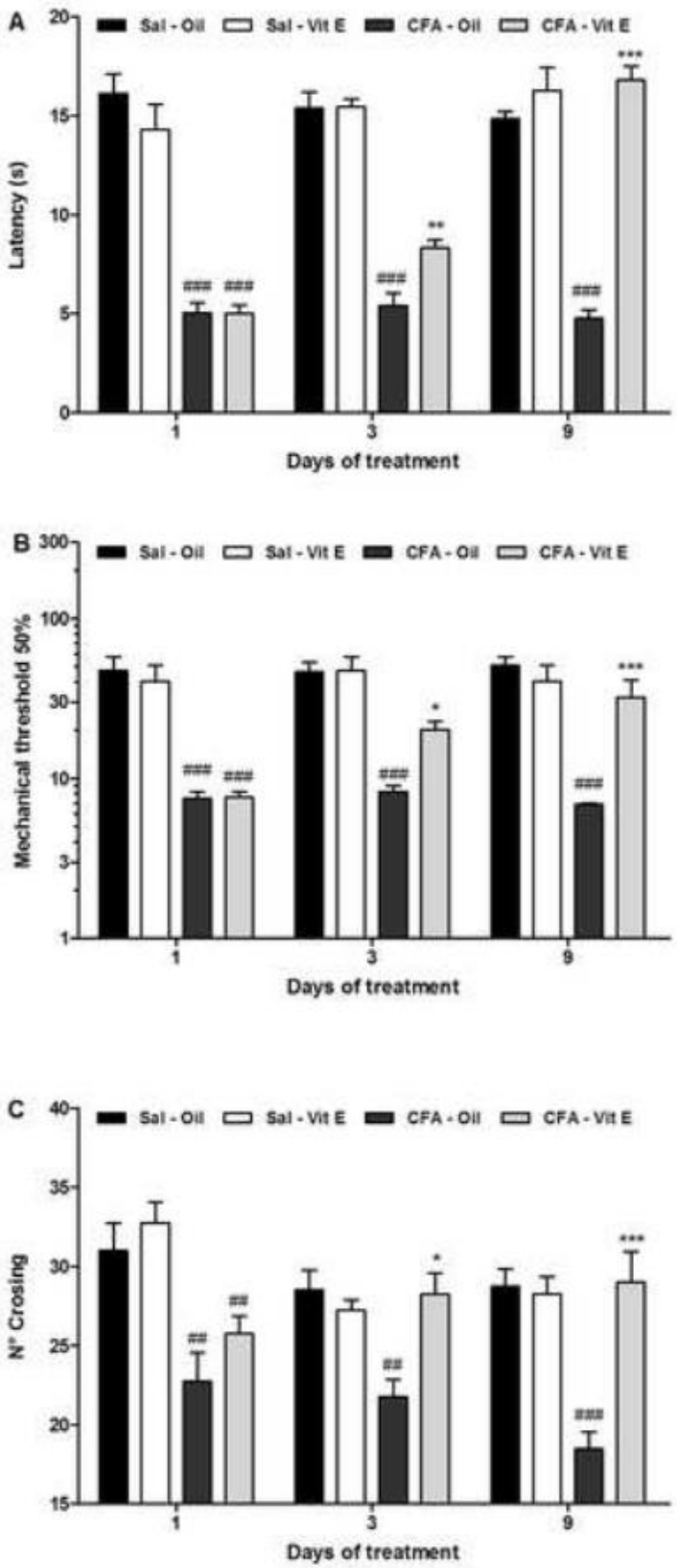

A
Sal - Oil
Sal - Vit E
CFA - Oil
CFA - Vit E
Latency (s)
20
15
10
5
0
###
**
***
1
3
9
Days of treatment
B
Mechanical threshold 50%
300
100
30
10
3
1
*
C
N° Crosing
40
35
30
25
20
15
##

Figure 4:

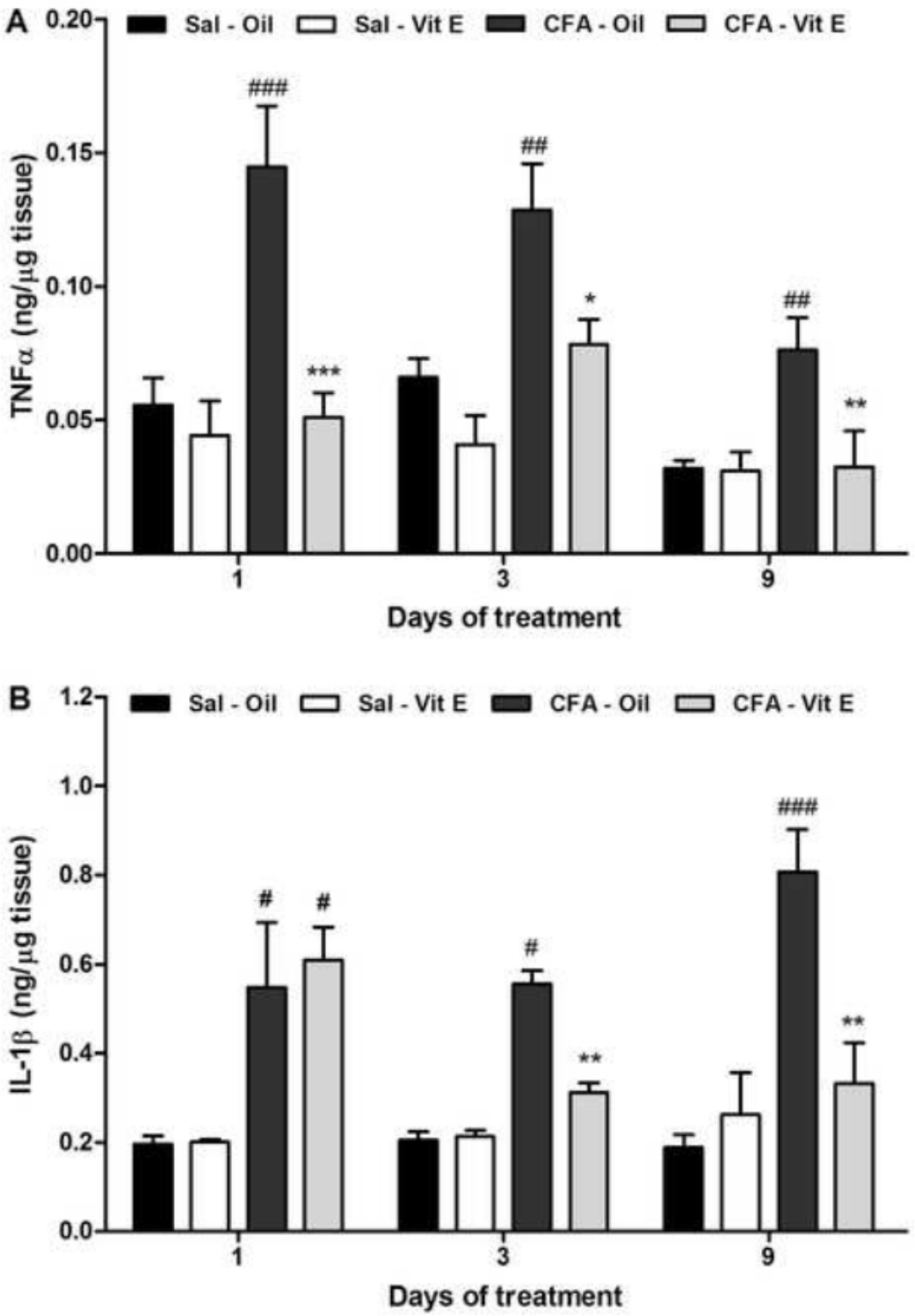
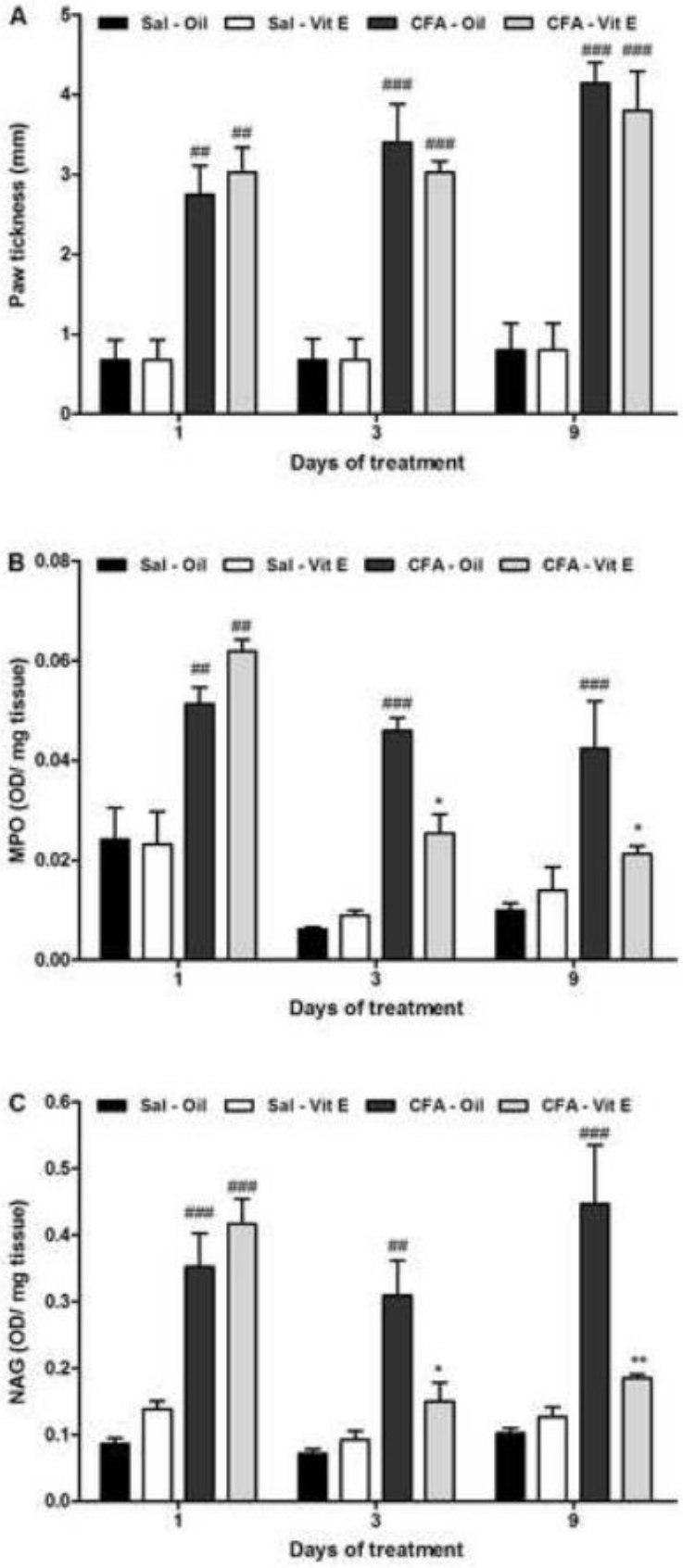

A
Sal - Oil
Sal - Vit E
CFA - Oil
CFA - Vit E
TNFα (ng/μg tissue)
0.20
0.15
0.10
0.05
0.00
###
##
*
***
##
**
1
3
9
Days of treatment
B
Sal - Oil
Sal - Vit E
CFA - Oil
CFA - Vit E
IL-1β (ng/μg tissue)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
#
#
#
**
###
**
1
3
9
Days of treatment

Figure 5:


A
Sal - Oil
Sal - Vit E
CFA - Oil
CFA - Vit E
Paw tickness (mm)
Days of treatment
B
MPO (OD/ mg tissue)
Days of treatment
C
NAG (OD/ mg tissue)
Days of treatment

6. DISCUSSÃO

A dor é um dos principais sintomas clínicos, característico de diversas patologias, sendo descrita como uma experiência sensorial e/ou emocional desagradável, associada ou não a uma lesão tecidual real ou potencial (Loeser e Treede, 2008). A pesar disso, a dor ainda apresenta diversas limitações de tratamento devido a falta de eficácia e desenvolvimento de efeitos adversos que limitam ou impossibilitam sua utilização (Milano et al., 2008).Com o intuito de ampliar nosso conhecimento sobre a neurotransmissão da dor, diversos grupos pesquisaram por alterações bioquímicas durante esse processo, e relataram o desenvolvimento de estresse oxidativo em diferentes tecidos durante esse evento (Vasanthi et al., 2009). Além disso, o efeito antinociceptivo/analgésico e anti-inflamatório de compostos com atividade antioxidante relatado em diferentes modelos de dor (Rossato et al., 2010; Zhao et al., 2014).

Assim, acreditamos que de algum modo ocorra à produção de RL durante a nocicepção, e isso contribua para a transmissão do estimulo doloroso. Sabemos que o NO, um dos principais RL produzidos durante a nocicepção, é capaz de interagir diretamente com alguns alvos moleculares, dentre eles o TRPV1 *in vitro* (Miyamoto et al., 2009). Devido a isso, nessa tese objetivamos investigar a possibilidade de ER, especialmente o NO, produzidas endogenamente durante a nocicepção, participarem da transmissão de estímulos dolorosos. Também investigamos a participação de compostos tiólicos exógenos e endógenos nesse processo, bem como a participação do receptor TRPV1.

Para isso inicialmente selecionamos um antioxidante usado clinicamente, a NAC, e investigamos seu efeito antinociceptivo. Observamos

que a NAC apresenta efeito antinociceptivo quando administrado tanto por via intraperitoneal quando por via intratecal, no teste da capsaicina, mas não por via local. Para definir qual dos sítios seria mais importante para seu efeito antinociceptivo, realizamos a administração intraplantar de NAC, sem observar efeito algum, e também realizamos a administração sistêmica (intraperitoneal) de NAC no teste da capsaicina intratecal. Neste protocolo observamos que a NAC apresentou efeito antinociceptivo. Como a NAC apresentou efeito antinociceptivo apenas quando administrada por uma via capaz de atingir o sistema nervoso central (medula espinhal), concluir que a medula espinhal parece ser o sítio mais relevante para o efeito antinociceptivo da NAC.

Concomitantemente, este modelo de nocicepção foi acompanhado pelo desenvolvimento de estresse oxidativo na porção lombar da medula espinhal dos camundongos, caracterizado pela diminuição dos níveis totais de tióis não proteicos, elevação dos níveis de TBARS e também de 3-NT. Além de diminuir a nocicepção induzida pela capsaicina, tanto o tratamento intraperitoneal quanto intratecal com NAC foi capaz de prevenir estas alterações. Dessa forma demonstramos que a nocicepção é acompanhada de uma alteração no estado redox da medula espinhal, e que a modulação deste estado pode ser um método para a modulação da dor. De fato, outras substâncias com atividade antioxidante já tiveram seus efeitos antinociceptivo, inclusive suas atividades diretamente no sistema nervoso central, reforçando nossos dados (Kundu et al., 2012; El Boghdady et al., 2012; Veselinovic et al., 2014).

Apesar dos resultados positivos, o teste de nocicepção induzida pela capsaicina intraplantar não apresenta elevada correlação com patologias associadas a dor. Por isso, decidimos utilizar outros dois modelos com maior

relevância, a inflamação crônica induzida por adjuvante (mimetiza quadros inflamatórios crônicos como algumas artrites) e o modelo de incisão plantar (mimetiza dor pós-cirúrgica) (Ferreira et al., 2004; Pogatzki e Raja, 2003). No primeiro modelo, observamos que a NAC desencadeou efeito antinociceptivo quando administrada sistemicamente e centralmente (intratecal), mas não perifericamente (intraplantar). Ao mesmo tempo, a inflamação crônica promoveu uma diminuição dos níveis de SH espinhais, os quais foram restaurados pelo tratamento com NAC. Esses resultados reforçam os dados obtidos com o teste da capsaicina. Apesar disso, no teste da incisão plantar, não observamos efeito antinociceptivo da NAC, ao mesmo tempo em que o modelo não foi capaz de alterar os níveis de SH espinhais. Dessa forma, concluímos que nem todos os tipos de nocicepção são capazes de promover estresse oxidativo espinhal, e que este evento parece necessário para que a NAC exerça seu efeito antinociceptivo.

Para corroborar este fato, decidimos avaliar a premissa contrária, ou seja, se a diminuição das defesas antioxidantes facilitaria/induziria a nocicepção em camundongos. Para isso administramos BSO, um inibidor irreversível da enzima γ-glutamil-cisteína-sintetase, a enzima passo limitante na síntese de glutationa, o principal componente tiólico não proteico no sistema nervoso central (Drake et al., 2002). Após a administração de BSO, observamos uma redução significativa nos níveis de tióis não proteicos espinhais, o qual foi acompanhado do desenvolvimento de hiperalgesia química, mecânica térmica, e alodínia mecânica. Dessa forma, concluímos que os níveis de SH espinhais apresentam grande relevância para o controle da dor. Similarmente, pacientes que apresentam deficiência na síntese de

glutationa desenvolvem, dentre outros sintomas, neuropatia periférica (Ristoff e Larsson, 2007).

A partir desse primeiro conjunto de resultados, concluímos que o estresse oxidativo produzido pela nocicepção, bem como os níveis espinhais de SH, parece importante para a transmissão de estímulos nociceptivos, e que a medula espinhal parece ser um sítio relevante para esse evento. Também, a NAC parece ser um antioxidante interessante para a utilização como adjuvante no tratamento da dor, uma vez que se mostrou capaz de atuar diretamente na medula espinhal, de forma segura, e desencadear efeito antinociceptivo e antioxidante.

Apesar disso, o exato mecanismo pelo qual o estresse oxidativo e compostos tiólicos participam da transmissão nociceptiva na medula espinhal ainda permanecia uma incógnita. Para tentar elucidar isso, decidimos investigar a possibilidade de que o NO, uma espécie reativa de nitrogênio extremamente importante na modulação da neurotransmissão central, poderia funcionar como um ativador direto do receptor TRPV1 *in vivo*, uma vez que essa possibilidade já foi apontada em experimentos *in vitro* (Yoshida et al., 2006; Miyamoto et al., 2009).

Para isso, investigamos inicialmente se o L-NAME, um inibidor da enzima responsável pela síntese de NO, apresentaria antinocicepção no teste da capsaicina, bem como a NAC. Como esperado, observamos que ambos apresentaram efeito antinociceptivo (anti-hiperalgésico) no teste da hiperalgesia térmica induzida por capsaicina intraplantar. Para corroborar esses resultados, induzimos o desenvolvimento de nocicepção (hiperalgesia térmica) pela administração intratecal de L-arginina, a qual também foi revertida pela

administração prévia de NAC e L-NAME. Como vimos anteriormente que o estresse oxidativo e os níveis de SH são importantes para a nocicepção na medula espinhal, decidimos investigar se tanto a administração de capsaicina intraplantar quanto a de L-arginina intratecal alterariam os níveis de tióis não proteicos e NOx na porção lombar da medula espinhal de camundongos. Como esperado, ambos os modelos alteraram os parâmetros oxidativo avaliados, apoiando nossa hipótese. Além disso, os tratamentos com NAC e L-NAME preveniram essas alterações. Já foi demonstrado que os SH espinhais são capazes de neutralizar o NO diretamente (Derakhshan et al., 2007).

Assim, é possível que durante a transmissão espinhal da dor, ocorra um aumento na produção de NO, suplantando as capacidades de neutralização dos SH presentes no tecido. Dessa forma, o NO poderia interagir com algum alvo farmacológico e facilitar a transmissão do estímulo nociceptivo. Por isso, quando administramos NAC conseguimos neutralizar esse excedente de NO e impedir a nocicepção; quando induzimos uma diminuição nos níveis espinhas de SH com BSO, os níveis basais de NO perdem seu “controle” e ficam livres para interagir com seus alvos.

Assim, observamos que o NO parece ser importante na nocicepção espinhal, e que a modulação de seus níveis (observado pela quantificação de NOx) parece ser relevante. Apesar disso, o mecanismo pelo o qual o NO participa desse processo ainda é incerto. Por isso decidimos investigar se o TRPV1 estaria envolvido nesse processo. Para isso, utilizamos três abordagens diferentes: bloqueio farmacológico do receptor utilizando um antagonista seletivo (SB366791), desfuncionalizando o receptor pela administração intratecal de uma elevada dose de agonista (capsaicina 2%) e

pela ablação gênica do receptor pela administração repetida de um oligonucleotídeo anti-sentido. Como resultado, observamos que os três protocolos foram capazes de bloquear parcialmente a resposta nociceptiva induzida pela capsaicina intraplantar. Acreditamos que isso se deva ao fato de que como administramos a capsaicina em um sitio diferente do tratamento (pata), outros mecanismos sejam ativados, que além da ativação espinhal do TRPV1. Apesar disso, observamos que os três protocolos foram capazes de abolir a resposta nociceptiva (hiperalgésica) desenvolvida pela administração intratecal de L-arginina. Isso reforça a ideia de que o NO produzido a partir da L-arginina tenha como alvo o TRPV1.

Para confirmar esses fatos, testamos ainda o efeito antinociceptivo da NAC, L-NAME e SB366791 nos modelos de nocicepção (hiperalgesia) induzida pela administração intratecal de BSO, nitroglicerina (NTG – doador de NO) e SIN1 (doador de peroxinitrito). Observamos que tanto a NAC quanto o SB366791 apresentaram efeito antinociceptivo nesses modelos, enquanto que o L-NAME inibiu apenas a nocicepção induzida pela BSO. Acreditamos que isso se deva ao fato de o efeito antinociceptivo do L-NAME se dever a inibição da NOS, e nesses dois casos (NTG e SIN1) as radicais livres estariam sendo liberadas no sistema de forma independente da NOS.

Por muito tempo, o TRPV1 foi apontado como um dos grandes pontos de modulação da nocicepção, o que impulsionou a busca por antagonistas seletivos. Apesar disso, essa busca se mostrou infrutífera, pois muitos antagonistas não apresentaram efeitos em humanos, e outros desenvolveram efeitos adversos severos, como hipertermia resistente a fármacos (Gavva et al., 2008). Assim, é possível crer que o bloqueio direto e total do receptor não seja

uma abordagem viável para o tratamento da dor, mas a possibilidade de impedirmos a sua super-ativação parece viável e frutífera. Dessa forma, com o grupo de dados que apresentamos nessa tese, podemos concluir que os compostos tiólicos desencadeiam efeito antinociceptivo em roedores, e que isso parece se dever ao fato de estes neutralizarem o NO formado durante a nocicepção. Assim, NO não poderia realizar a ativação do TRPV1 espinhal, bloqueando a transmissão da dor.

Uma vez que investigamos as atividades antinociceptiva da NAC e seu possível mecanismo de ação, decidimos posteriormente investigar o efeito de outro antioxidante, a vit-E. Como ela já foi testada de forma crônica em diferentes patologias inflamatórias crônicas em humanos, sem resultados conclusivos, decidimos avaliar seus possíveis efeitos em um modelo de inflamação crônica, em ratos. Para isso, selecionamos o modelo de inflamação crônica induzida por adjuvante, uma vez que esse mimetiza várias alterações características de diversos tipos de artrite (Pisetsky e Ward, 2012). Também, como os pacientes nestes relatos utilizaram doses repetidas de vit-E, decidimos investigar seu efeito após diferentes dias de tratamento sistêmico.

Inicialmente investigamos seu provável efeito global sobre as alterações observadas através de um escore de artrite. A partir desse tipo de análise, observamos que o tratamento repetido com vit-E desencadeia um efeito “tipo-anti-artrítico” a partir de três dias de administração, revertendo parcialmente as alterações induzida pelo adjuvante. Esse efeito se intensificou atingindo seu ápice após nove dias de tratamento. Esse padrão de resposta com inicio retardado foi observado também em outros trabalhos que descreveram efeitos antinociceptivo da vit-E (Kin et al., 2004). Esse fato provavelmente se deva pela

necessidade de um acúmulo de vit-E no tecido para desencadear seus efeitos. Esse fato pode ser comprovado pela observação de que o tratamento com vit-E é capaz de induzir aumentos significativos nos níveis de vit-E a partir do terceiro dia, tanto no tecido plantar quando no soro, a partir do terceiro dia.

Acreditamos que essa alteração global no processo inflamatório se deva a atividade antioxidante apresentada pela vit-E. De forma similar, ocorreu um aumento nos níveis teciduais e plasmáticos de vit-E a partir do terceiro dia de tratamento, mesmo período no qual observamos as primeiras alterações no escore de artrite. Ainda, a injeção de adjuvante foi capaz de promover uma diminuição nos níveis teciduais de vit-E, sugerindo o desenvolvimento de um estresse oxidativo no sítio da inflamação.

Tendo visto isso, decidimos avaliar os efeitos desencadeados pela vit-E em três tempos distintos de tratamento: um dia, três dias e nove dias de tratamento, nos quais observamos nenhum efeito, inicio do efeito e ápice do efeito, respectivamente. Nestes tempos, observamos que o tratamento foi capaz de desencadear também uma resposta antinociceptiva (anti-hiperalgésica e anti-alodínica), bem como recuperar a funcionalidade do membro afetado. Sabe-se que o peróxido é capaz de promover a ativação e sensibilização de nociceptores e outras terminações nervosas (Abe et al., 1998; Trevisan et al., 2013). Dessa forma, é possível que a elevação nos níveis de peróxido seja capaz de promover deflagração das primeiras alterações sensoriais (hiperalgesia e alodínia).

Dessa forma, é possível que o acúmulo de vit-E no sítio da inflamação seja capaz de neutralizar o excesso de peróxido produzido, prevenindo/revertendo à ativação/sensibilização desses nociceptores,

desencadeando uma resposta antinociceptiva. Isso é muito relevante, uma vez que esses são os principais sintomas que indicam a presença da doença e levam os pacientes a buscarem atendimento médico. Clinicamente, esses são os sintomas que mais debilitam o paciente e afetam sua qualidade de vida, uma vez que diminuem a capacidade de desenvolver as atividades cotidianas (Pisetsky e Ward, 2012).

Apesar disso, é necessário avaliar outros sintomas relevantes a um processo inflamatório, como infiltração celular, e produção de citocinas inflamatórias. Esses elementos são muitas vezes os responsáveis pela promoção da lesão tecidual e manutenção da doença (Aryaeian et al., 2008). Similarmente, observamos também uma diminuição progressiva do processo de infiltração celular (MPO – neutrófilos e NAGase – macrófagos), bem como dos níveis teciduais de interleucinas (TNFα e IL1β). Esse fato também pode ser explicado pela neutralização de espécies reativas, como o peróxido de hidrogênio. Já foi demonstrado que este pode funcionar tanto como fator quimiotático, estimulando o processo de infiltração celular, bem como ativar vias de sinalização responsáveis pela produção e liberação de citocinas pró-inflamatórias (Woolf et al., 1997; van der Vliet e Janssen-Heininger, 2014).

Dessa forma, de modo geral, podemos concluir que a vit-E de fato apresenta um efeito antinociceptivo em modelo de inflamação crônica. Este efeito parece estar relacionado com a capacidade de neutralização de espécies reativas, como o peróxido de hidrogênio produzido durante esse processo. A partir desses resultados, é possível apontar uma potencial utilização clínica de vit-E durante essas patologias (artrites), não como uma monoterapia, mas sim

como um adjuvante terapêutico, podendo ainda ser incorporado a diferentes formulações de fármacos tradicionalmente utilizados.

7. CONCLUSÕES

No decorrer deste trabalho, objetivamos investigar a possibilidade de radicais livres produzidas endogenamente participarem da gênese e transmissão de estímulos dolorosos. Trabalhamos com esse intuito a fim de esclarecer os eventos da transmissão central e periférica da dor e apontar possíveis alvos farmacológicos para a modulação desse evento e possíveis tratamentos (Figura 3). A partir dos resultados apresentados neste trabalho, concluímos:

1. O antioxidante NAC apresenta efeito antinociceptivo em diferentes modelos de nocicepção em camundongos. Este efeito parece estar relacionado com a neutralização de radicais livres produzidas na medula espinhal após a estimulação nociva periférica;
2. Diferentes tipos de nocicepção periférica e central são capazes de alterar marcadores de estresse oxidativo espinhais, como diminuição dos níveis de SH, aumento dos níveis de TBARS, 3-NT e NOx;
3. A diminuição das defesas antioxidantes na medula espinhal (diminuição dos níveis de SH) torna os camundongos mais sensíveis à estimulação periférica;
4. O aumento dos níveis de NOx na medula espinhal leva a uma diminuição dos níveis de SH e a nocicepção dependente do TRPV1;
5. Observamos que o tratamento repetido com vit-E é capaz de reverter diferentes parâmetros nociceptivos induzidos por inflamação crônica em ratos;
6. O tratamento repetido com vit-E também consegue reverter diversos parâmetros inflamatórios induzidos;

7. Tanto a atividade antinociceptiva quanto anti-inflamatória da vit-E parece estar relacionada com a sua atividade antioxidante e capacidade de neutralizar o excesso de H_2O_2 produzido durante a inflamação crônica.

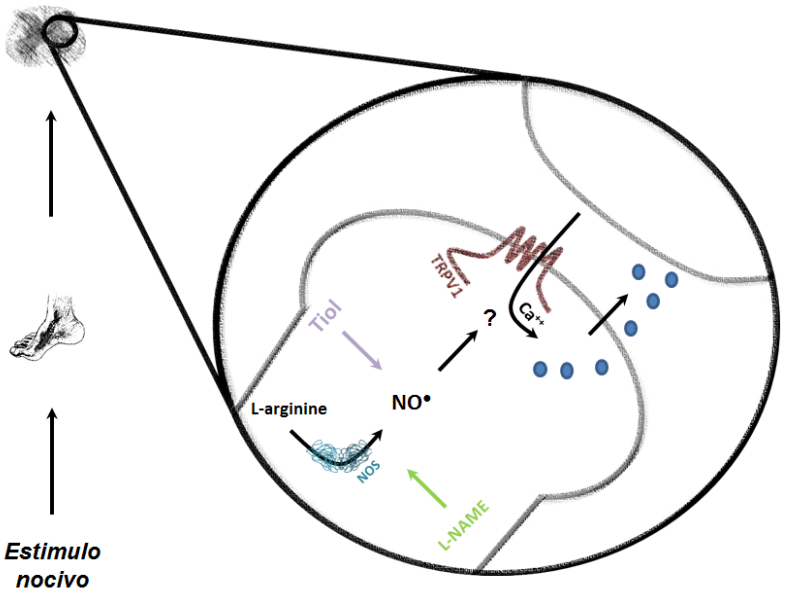


Figura 3: Representação esquemática da conclusão desta tese: A estimulação nociceptiva periférica induzindo um aumento nos níveis espinhais de NO, os quais podem atuar como ativador endógeno do TRPV1. Além disso, esses níveis de NO podem ser modulados por compostos tiólicos não proteicos, tanto endógenos quanto exógenos.

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