Universidade Estadual de Londrina

CAMILA RODRIGUES FERRAZ

MECANISMOS PERIFÉRICOS ENVOLVIDOS NA HIPERALGESIA INDUZIDA PELA JARARAGINA:

PARTICIPAÇÃO DAS CITOCINAS TNF-α E IL-1β E DO FATOR DE TRANSCRIÇÃO NFκB

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Dissertação apresentada ao Programa de Pós Graduação em Ciências da Saúde do Centro de Ciência da Saúde da Universidade Estadual de Londrina

Orientador: Waldiceu Aparecido Verri Junior.

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Londrina, 16 de março de 2015**.**

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FERRAZ, Camila Rodrigues. **Mecanismos periféricos envolvidos na hiperalgesia induzida pela jararagina em camundongos:** participação das citocinas TNF-α e IL-1β e do fator de transcrição NFκB. 2015. 68 f. Dissertação (Mestrado em Ciências da Saúde) – Centro de Ciências da Saúde, Universidade Estadual de Londrina, Londrina, 2015.

RESUMO

A Jararagina é uma metaloproteinase hemorrágica presente no veneno da *Bothrops jararaca*, cujo efeito nociceptivo e mecanismos nociceptivos com enfoque na participação das citocinas pró-inflamatórias TNF-α e IL-1β e do fator de transcrição (NFκB) foram investigados. A administração intraplantar de jararagina (1, 10, 100 e 1000 ng/pata) induziu hiperalgesia mecânica e aumentou os níveis de TNF-α 1ª,3ª e 5ª hora e IL-1β na 0,5ª, 1ª e 3ª hora após sua administração. O pré-tratamento com a morfina (2, 6, 12 µg/paw) inibiu a hiperalgesia mecânica induzida pela jararagina. O pré-tratamento sistêmico ou local com Etanercept (10 mg/Kg; 100 µg/pata), IL-1ra (30 mg/Kg; 100 pg/pata) e PDTC (100 mg/Kg; 100 µg/pata), respectivamente, inibiram a hiperalgesia mecânica induzida pela jararagina. O pré-tratamento com PDTC diminuiu os níveis de TNF-α e IL-1β produzido pela administração da jararagina. A co-administração de jararagina (0,1 ng/pata) com TNF-α (0,1 pg/pata) ou jararagina (0,1 ng/pata) com IL-1β (1 pg/pata) demonstrou potencialização da hiperalgesia mecânica comparada a administração separada de jararagina, TNF-α e IL-1β. Dessa forma, este estudo demonstrou o envolvimento das citocinas pró-inflamatórias TNF-α e IL-1β e do fator de transcrição NFκB na hiperalgesia mecânica induzida pela jararagina.

Palavras-chave: Jararagina. Metaloproteinase. Citocina. Fator de transcrição nuclear. Hiperalgesia.

FERRAZ, Camila Rodrigues. **Peripheral mechanisms involved in jararhagin-induced mechanical hyperalgesia in mice:** participation of the cytokines TNF-α and IL-1 β, and the transcription factor NFκB. 2015. 68 p. Dissertação (Mestrado em Ciências da Saúde) – Centro de Ciências da Saúde, Universidade Estadual de Londrina, Londrina, 2015.

ABSTRACT

Jararhagin is hemorrhagic metalloprotease isolated from *Bothrops jararaca* snake venom. In the present study, the participation of prohyperalgesic cytokines (TNF-α and IL-1β) and transcription factor (NFκB) in jararhagin-induced mechanical hyperalgesia were evaluated. Intraplantar administration of jararhagin (1, 10, 100 and 1000 ng/paw) induced mechanical hyperalgesia and increased the levels of TNF-α 1, 3 and 5 h, and IL-1β levels at 0.5, 1 and 3 hour after its administration. The pre-treatment with morphine (2, 6, 12 µg/paw) inhibited jararhagin-induced mechanical hyperalgesia. The systemic or local pre-treatment with Etanercept (10 mg/Kg; 100 µg/paw), IL-1ra (30 mg/Kg; 100 pg/paw) and PDTC (100 mg/Kg; 100 µg/paw), respectively, inhibited jararhagin-induced mechanical hyperalgesia. The pre-treatment with PDTC decreased the levels of TNF-α and IL-1β produced by the administration of jararhagin. Co-administration of jararhagin (0,1 ng/paw) plus TNF-α (0,1 pg/paw) or jararhagin (0,1 ng/paw) plus IL-1β (1 pg/paw) demonstrated a potentiating hyperalgesic response of those combinations. Thus, the present study demonstrated the involvement of pro-hyperalgesic cytokines TNF-α and IL-1β, and nuclear transcriptions factor NFκB in jararhagin-induced mechanical hyperalgesia in mice.

Keywords: Jararhagin. Metalloprotease. Cytokines. NFκB. Hyperalgesia.

SUMÁRIO

1 INTRODUÇÃO

A capacidade de detectar estímulos potencialmente nocivos (nociceptivos) é conferida pelo sistema somatosensorial, e envolve interações complexas entre mecanismos periféricos e centrais. A detecção de estímulos nocivos depende da ativação dos neurônios nociceptivos ou nociceptores, que são amplamente distribuídos pelo corpo (pele, músculos, articulações, vísceras e meninges). As fibras nociceptivas englobam as fibras A-delta que transduzem estímulos mecânicos e as fibras C que transduzem estímulos mecânicos, térmicos ou químicos em impulsos elétricos que serão então transmitidos ao sistema nervoso central (Macintyre et al., 2010).

A lesão tecidual associada à infecção, inflamação ou isquemia resulta na liberação de inúmeros mediadores, dentre os quais as prostaglandinas e as aminas simpáticas desempenham papel proeminente (Ferreira and Nakamura, 1979, Khasar et al., 1999). Esses mediadores interagem preferencialmente com receptores metabotrópicos (receptores que não estão ligados diretamente aos canais iônicos, mas às vias metabólicas de sinalização intracelular) expressos na membrana neuronal das fibras C, associadas à condução da dor inflamatória (Schaible and Schmidt, 1988). A interação desses mediadores com seus respectivos receptores resulta na liberação de mensageiros secundários, tais como a adenosina 3’ 5’ monofosfato cíclico (AMPc), proteína quinase A (PKA) e proteína quinase C (PKC). Consequentemente, ocorre a fosforilação de canais de sódio dependentes de voltagem (Nav1.8) e a inibição de canais de potássio, que diminuem o limiar de disparo de potenciais de ação dos neurônios aumentando sua excitabilidade (revisado por Ferreira et al., 2008).

Estímulos inflamatórios como (lipopolissacarídeo) LPS e carragenina induzem a liberação de uma cascata hierárquica de citocinas, que por sua vez promovem a liberação de mediadores tais como prostaglandinas e aminas simpáticas (Cunha et al., 2005, Verri et al.,2006a). O estímulo inflamatório é reconhecido por células residentes tais como macrófagos e mastócitos que liberam diferentes citocinas e quimiocinas, com papel chave no desencadeamento da dor inflamatória. A primeira evidência experimental da participação de citocinas na dor inflamatória foi obtida por Ferreira e colaboradores em 1988. Neste trabalho, foi demonstrado que injeção intraplantar de interleucina-1β (IL-1β) induz hiperalgesia mecânica severa, dependente da liberação de prostanoides. Posteriormente a este achado, inúmeras citocinas, quimiocinas e outras moléculas, foram descritas como componentes de uma cascata de mediadores responsáveis pela liberação de prostaglandinas, aminas simpáticas, endotelinas, entre outros, que ativam diretamente os nociceptores, gerando um panorama de sinalização celular específico para cada doença ou modelo experimental (Verri et al., 2006a).

O fator de necrose tumoral-alfa (TNF-α) desempenha papel chave no desencadeamento da dor inflamatória (Cunha *et al.*, 1992). Diferentes estímulos inflamatórios como o LPS e a carragenina induzem a liberação de bradicinina que estimula a liberação do TNF-α. O TNF-α induz a produção de IL-6 e IL-1β, que estimulam a formação de produtos da ciclooxigenase através da indução enzimática da ciclooxigenase-2 (COX-2), resultando principalmente na produção de prostaglandina E2, elemento fundamental para o estabelecimento da hiperalgesia inflamatória. O TNF-α também é capaz de induzir a liberação de quimiocinas (IL-8/CXCL8 em humanos, CINC-1 em ratos), que estimulam a liberação/produção de aminas simpáticas (Cunha et al.,1991, Cunha et al.,1992, Ferreira et al.,1988, Ferreira et al.,1993a, Ferreira et al.,1993b, Lorenzetti et al.,2002).

Outras citocinas também são importantes na gênese da hiperalgesia inflamatória de origem imune. As citocinas IL-15 e IL-18 são cruciais para o desenvolvimento da hiperalgesia em modelos de inflamação tipo Th1. O desafio com ovalbumina em camundongos imunizados resulta na liberação de IL-15 e IL-18, que induzem a produção sequencial de interferon-γ, endotelina-1, culminando na síntese de prostaglandina E2 (Verri et al.,2007, Verri et al.,2006b). Posteriormente, a participação de citocinas pleiotrópicas IL-33 e IL-17, na hiperalgesia de origem imune também foi descrita. A IL-33 é capaz de mediar a hiperalgesia cutânea e articular através da liberação sequencial de TNF-α, IL-1β, IFN-γ, endotelina-1 e prostaglandinas (Verri et al., 2008). A IL-17 foi descrita como uma importante citocina pró-nociceptiva liberada em modelo de artrite induzida por antígeno. O mecanismo de ação da IL-17 é dependente de neutrófilos, citocinas (TNF-α, IL-1β), quimiocinas, metaloproteinases de matriz extracelular (MMPs), endotelinas, prostaglandinas, e aminas simpáticas (Pinto et al., 2010). Esses achados refletem a natureza multifatorial da dor de origem inflamatória que é mediada por diferentes citocinas, que atuam paralelamente, em sequência ou até mesmo em sinergismo (Verri et al., 2008). É importante ressaltar a participação do TNF-α e IL-1β na maioria dos modelos de dor e seu papel como alvos terapêuticos comprovados clinicamente (Verri, et.al., 2006a).

Durante o processo inflamatório, além da produção de citocinas pró-nociceptivas, também ocorre a liberação de outras citocinas, capazes de modular negativamente o processo inflamatório e consequentemente, a dor inflamatória, sendo consideradas anti-nociceptivas. As principais são IL-4, IL-10, IL-13 e o antagonista de receptor da IL-1 (IL-1ra), uma molécula endógena que limita a disponibilidade de receptores para a IL-1α e IL-1β reduzindo a ativação pró-inflamatória celular (revisado por Verri et al., 2006a).

Outros mecanismos também são responsáveis pela regulação de citocinas pró-nociceptivas/inflamatórias. O fator de transcrição nuclear NFκB, por exemplo, é uma peça chave no controle da imunidade inata e adaptativa. Ele está presente no citoplasma em associação com proteínas inibitórias conhecidas como inibidores do NFκB (IκB). Após a

ativação celular, por exemplo por citocinas ou agonistas de receptores tipo toll, o IκB é fosforilado e sofre degradação pelo sistema proteassoma, o que culmina na translocação do NFκB para o núcleo, onde regula a transcrição de diversos genes, tais como citocinas, quimiocinas, moléculas de adesão, MMPs, ciclooxigenase 2 (COX-2) e óxido nítrico sintase (iNOS). Além disso, alguns trabalhos demonstram que o TNF-α e IL-1β também podem induzir a fosforilação e ativação do NFκB independente da degradação do IκB (Li and Verma, 2002).

Paralelamente, a ativação de diferentes vias de sinalização (MAPK e PI_3K) pode estar envolvida na hiperalgesia através da modulação de canais iônicos, aumento da produção de citocinas, e outros mediadores que resultam na sensibilização de nociceptores (Gao and Ji, 2010). Recentemente, foi demonstrado que a hiperalgesia induzida pelo fator estimulador de colônias granulocitárias (G-CSF) é mediada por MAPK (ERK, JNK, p38) e PI_3K (Carvalho et al., 2011). Outras moléculas, tais como as MMPs (Kawasaki et al., 2008) e caspase-1 (Cunha et al., 2010), também estão envolvidas no desenvolvimento da dor neuropática, e na gênese na hiperalgesia inflamatória induzindo a maturação da IL-1β, respectivamente. Desta forma, uma miríade de moléculas participam da gênese e da regulação da dor inflamatória. O entendimento desses mecanismos complexos pode ser útil para identificação de novos alvos de ação de drogas que visam combater/minimizar a dor de diferentes etiologias.

Dor e inflamação são associados ao envenenamento por serpentes botrópicas, responsáveis pela maioria dos acidentes ofídicos no Brasil (Ministério da Saúde, 2014; Gutierrez et al.,1998, Gutierrez and Rucavado, 2000). Associando-se a estes sintomas, hemorragia, edema e mionecrose se instalam no local da picada, resultando em sérias complicações clínicas, como sequelas permanentes ou até mesmo a amputação do membro afetado (Gutierrez et al., 1998). O tratamento das vítimas é realizado pela administração da soroterapia, que neutraliza com eficiência os efeitos sistêmicos do envenenamento, tais como alterações na coagulação sanguínea e cardiovasculares, choque hipovolêmico e alterações renais. No entanto, os efeitos locais, como hemorragia, necrose (Cardoso et al., 1993), hiperalgesia e edema (Picolo et al., 2002) não são neutralizados com eficiência. Diante disso, estudos que visam elucidar os mecanismos envolvidos na dor e inflamação induzidos pelo envenenamento são importantes para desenvolvimento de estratégias terapêuticas que visam melhorar o tratamento das vítimas de acidentes ofídicos.

O primeiro relato sobre o efeito hiperalgésico de venenos botrópicos foi descrito em 1994 (Teixeira et al., 1994). Nesse trabalho, verificou-se que a hiperalgesia induzida pelo veneno de *Bothrops jararaca*, serpente amplamente distribuída nas regiões Sul e Sudeste do país, é mediada por prostagladinas, leucotrienos e fator de agregação plaquetária (PAF). Posteriormente, a participação de bradicinina (Chacur et al., 2001, Chacur et al., 2002) e

aminas biogênicas (Rocha et al., 2000) também foi relatada. Estudando os componentes isolados do veneno de *Bothrops asper*, verificou-se que as fosfolipases A2 miotóxicas desempenhavam papel crucial na indução da hiperalgesia (revisado por Teixeira et al., 2009). A hiperalgesia induzida pela MT-I e MT-II, miotoxinas isoladas do veneno *B. asper* é mediada por aminas biogênicas, leucotrienos e citocinas (TNF-α e IL-1β) (Chacur et al., 2003). Além disso, a administração periférica e periciática dessas duas miotoxinas também induz sensibilização da medula espinhal, através da liberação de citocinas, prostanoides e óxido nítrico (Chacur et al., 2004a, Chacur et al., 2004b), o que além de demonstrar o envolvimento de mecanismos espinais ativados por estímulo periférico, sugere a possibilidade do controle espinal da dor induzida pelo veneno, que não é neutralizada pelo tratamento com soro antiofídico. Um mecanismo alternativo que poderia acentuar a hiperalgesia induzida por fosfolipases A2 miotóxicas foi recentemente descrito (Cintra-Francischinelli et al., 2010). Nesse trabalho, foi relatado que miotoxinas (MT-I e MT-II) induziram liberação de ATP e íons potássio, potentes estimuladores de neurônios periféricos, em cultura de células musculares e em biópsias de músculos. Esse mecanismo poderia contribuir para disseminação da dor e lesão muscular induzidas por essas toxinas.

Outra importante classe de toxinas envolvidas na patologia local do envenenamento são as metaloproteinases de veneno de serpentes (SVMPs). As SVMPs compreendem uma série de enzimas dependentes de zinco, que são encontradas em grande quantidade nos venenos botrópicos (Fox and Serrano, 2005). As SVMPs representam cerca de 51.5% do veneno de *B. jararaca* (Fox et al., 2006), enquanto que as miotoxinas possuem composição minoritária (Moura-da-Silva et al., 1991). Interessantemente, foi descrito que as SVMPs são as principais responsáveis pela hiperalgesia induzida pelo veneno de *B. jararaca*, e que miotoxinas e serinoproteinases não contribuem significativamente para esse efeito (Zychar et al., 2010). Diante disso, o papel das SVMPs na hiperalgesia induzida por veneno de *B. jararaca* deve ser considerado.

O potencial hiperalgésico de SVMPs não foi muito explorado. Alguns trabalhos na década de 1990 relataram que as SVMPs não estavam envolvidas na hiperalgesia induzida pelo veneno de *B. jararaca* (Chacur et al., 2001, Chacur et al., 2002). Porém, estudos posteriores trouxeram novas evidências demonstrando o efeito hiperalgésico destas toxinas. A abordagem experimental da maioria destes estudos consistia na utilização de inibidores de SVMPs tais com o EDTA e a orto-fenantrolina, para inativar SVMPs presentes no veneno total. Desta forma, foi verificado que as SVMPs participavam efetivamente do desencadeamento da dor e inflamação induzidas pelo veneno de *B. jararaca* (Bonavita et al., 2006, Rocha et al., 2000, Zychar et al., 2010). A literatura descreve apenas dois trabalhos onde o efeito hiperalgésico de SVMPs isoladas foi estudado (Dale et al., 2004, Fernandes et al., 2007). A primeira evidência do efeito hiperalgésico de SVMPs foi descrito

para a jararagina, toxina hemorrágica mais abundante do veneno de *B. jararaca* (Dale et al., 2004). No entanto, os mecanismos/mediadores envolvidos nesse efeito não foram explorados. Fernandes e colaboradores (2007), demonstraram que a BaP1, SVMP isolada de *B. asper*, foi capaz de induzir hiperalgesia articular, num mecanismo dependente de prostanglandina E2 e TNF-α. Desta forma, novos estudos buscando avaliar o potencial hiperalgésico de SVMPs são importantes para identificar os principais mecanismos envolvidos neste efeito.

Considerando as evidências descritas acima, a jararagina representa uma boa ferramenta para estudar a hiperalgesia induzida pelas SVMPs. A jararagina é uma proteína de cadeia única, de 52 KDa, composta pelo domínio metaloproteinase, domínio tipo-disintegrina e pelo domínio rico em cisteínas (Paine et al., 1992). O domínio catalítico inclui o sítio de ligação com o zinco (HEBXHXBGBXH) e o motivo estrutural “met turn” que também estão presentes nas MMPs e estão envolvidos com a catálise de componentes de matriz extracelular (Bode et al., 1993). Seguindo-se ao domínio catalítico, o domínio tipo-disintegrina conserva os resíduos de cisteína nas mesmas posições que as disintegrinas clássicas que contêm o tripeptídeo funcional RGD, apresentando substituições nesses resíduos pela sequência SECD. Além disso, a jararagina possui a adição de um domínio carboxi-terminal rico em cisteínas, ao qual tem sido atribuídas propriedades adesivas à componentes plasmáticos e proteínas da matriz (Serrano et al., 2006). Além da similaridade estrutural com as MMPs, observada no domínio catalítico, a jararagina apresenta similaridade estrutural com as ADAMs, proteínas envolvidas em comunicação e regulação da função celular, principalmente nos domínios tipo-disintegrina e rico em cisteínas (Fox and Serrano, 2008).

Dentre as principais atividades biológicas da jararagina, destaca-se sua intensa atividade hemorrágica (Paine et al., 1992) correlacionada com a degradação de componentes plasmáticos (Kamiguti et al., 1994), afinidade e hidrólise de colágeno (Baldo et al., 2010, Moura-da-Silva et al., 2008) e inibição da agregação plaquetária (De Luca et al., 1995, Kamiguti et al., 1996). Em células endoteliais, a jararagina ativa a produção de óxido nítrico, prostaciclina e IL-8 (Schattner et al., 2005), entretanto, interfere com a adesão focal induzindo apoptose (Baldo et al., 2008, Tanjoni et al., 2003). A jararagina também possui evidente efeito pró-inflamatório. Essa toxina é capaz de processar a forma precursora do TNF-α na mesma posição que a TACE (TNF-α converting enzyme /ADAM17), liberando o TNF-α em sua forma biologicamente ativa *in vitro* (Moura-da-Silva et al., 1996). Estudos posteriores indicaram que a jararagina também induz a expressão de TNF-α, IL-6 e IL-1β em cultura de macrófagos (Clissa et al., 2001), e que a necrose induzida por essa toxina foi ausente em camundongos deficientes em TNFR1 e TNFR2, e parcialmente inibida em animais deficientes em IL-6 (Laing et al., 2003). Em outro estudo, (Gallagher et al., 2005)

verificaram um aumento significativo na expressão gênica de IL-1β, IL-6, TNF-α, CXCL1 (Chemokine-C-X-C-motif-ligand 1), CXCL2 (Chemokine-C-X-C-motif-ligand 2) e CXCL8 (Chemokine-C-X-C-motif-ligand 8) em fibroblastos e em tecido murino. A jararagina também induz migração leucocitária em camundongos, de forma dependente de macrófagos (Costa et al., 2002). No entanto, apesar da resposta inflamatória induzida pela jararagina ter sido muito explorada em algumas áreas, a relação dessa resposta com a indução de hiperalgesia não foi elucidada. Os dados anteriores obtidos por Dale e colaboradores (2004), mostraram apenas que a jararagina induziu hiperalgesia mecânica em ratos, mas não avançaram no sentido de estabelecer os mecanismos envolvidos neste efeito e sua correlação com a liberação de citocinas.

Uma vez que a dor é um importante sinal clínico observado em vítimas de acidentes ofídicos e em modelos experimentais nesses acidentes, a caracterização dos componentes/mecanismos envolvidos neste efeito é de extrema valia para o desenvolvimento de novas estratégias terapêuticas para melhorar o tratamento/recuperação das vítimas de acidentes ofídicos.

Nesse sentido, a jararagina mostra-se um bom modelo para estudar o efeito hiperalgésico das SVMPs. Uma vez que foi anteriormente demonstrado que a jararagina induz liberação de citocinas pró-inflamatórias e hiperalgesia mecânica, nossa hipótese considera que estes mediadores e/ou outros ainda não descritos poderiam ativar uma cascata de sinalização culminando na sensibilização de nociceptores, resultando em hiperalgesia inflamatória. Este aspecto ainda não foi abordado na literatura, e agregaria novas informações sobre a participação das SVMPs na hiperalgesia induzida pelos venenos ofídicos.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar a participação periférica das citocinas TNF-α e IL-1β e do fator de transcrição NFκB na hiperalgesia mecânica induzida pela jararagina.

2.2 OBJETIVOS ESPECÍFICOS

1. Avaliar se a jararagina induz hiperalgesia mecânica dose-dependente;
2. Avaliar a sensibilidade à morfina da hiperalgesia induzida pela jararagina;
3. Avaliar o perfil temporal da produção de TNF-α e IL-1β induzida pela administração de jararagina;
4. Avaliar se há participação do TNF-α, IL-1β e NFκB na hiperalgesia induzida pela jararagina com o tratamento com inibidores como etanercept, IL-1ra e PDTC.
5. Avaliar a participação do NFκB na produção de TNF-α e IL-1β induzida pela jararagina;
6. Avaliar se a hiperalgesia induzida pela jararagina é potencializada pelo TNF-α e IL-1β.

3 MATERIAL E MÉTODOS

3.1 ANIMAIS

Os experimentos foram realizados utilizando camundongos machos da linhagem Swiss (20 a 25g). Os camundongos Swiss foram provenientes do biotério central da Universidade Estadual de Londrina. Para realização dos experimentos foram selecionados grupos de 06 camundongos (ao acaso). Os camundongos ficaram no biotério em ciclo claro/escuro (12/12h), com livre acesso a água e ração. As amostras selecionadas tinham características similares, sendo da mesma raça, mesmo sexo, mesma idade e pesos próximos. Foram realizadas duas repetições para cada modelo experimental. Foi utilizado o n=06 de animais por grupo.

Todos os experimentos foram conduzidos de acordo com as normas estabelecidas pelo Comitê de Ética em Experimental Animal da Universidade Estadual de Londrina, CEUA- UEL n°7786.2014.42.

3.2 VENENO

O veneno de *Bothrops jararaca* foi obtido do "*pool"* de venenos extraídos de serpentes mantidas em cativeiro no biotério central do Laboratório de Herpetologia do Instituto Butantan, São Paulo-SP. As amostras de veneno foram liofilizadas e mantidas a -80°C até o momento do uso. A jararagina foi purificada conforme metodologia descrita por Paine e colaboradores (1992) e modificada por Moura-da-Silva e colaboradores (2003).

3.3 PROTOCOLOS EXPERIMENTAIS E TRATAMENTOS

A jararagina foi administrada via subcutânea intraplantar (i.pl.) nas doses de 1, 10, 100 e 1000 ng/pata, sendo escolhida a dose de 1000 ng/pata ou 1 µg/pata para os experimentos posteriores. Animais controles receberam a injeção i.pl com solução salina. Para avaliação da produção de citocinas, os camundongos foram eutanasiados com isofluorano inalatório e a coleta do tecido plantar de cada grupo experimental foi realizada nos tempos 1, 3 e 5 horas após o estímulo. A avaliação do efeito do PDTC na produção de citocinas foi realizado na 3° hora. Para a avaliação do tratamento com a morfina (agonista opióide) (Cristalia, São Paulo, Brasil), foi realizada um curva dose-resposta (2, 6, 12 µg/pata via i.pl.) 1 hora antes da administração de jararagina. Para a avaliação com os diferentes inibidores foi realizado o pré-tratamento com etanercept (Enbrel®, Wyeth Indústria Farmacêutica Ltda, São Paulo, Brazil), 10 mg/Kg, 200 µL, i.p., pré-tratamento 48 horas e 1

hora antes do estímulo ou 100 µg/pata, 20 µL, i.pl., pré-tratamento 1 hora antes da administração da Jararagina; IL-1ra (“National Institute of Biological Standards and Control”, UK), 30 mg/Kg, 200 µL, i.p. ou 100 pg/pata, 20 µL, i.pl., pré-tratamento 30 minutos antes do estímulo e PDTC (Santa Cruz Biotechnology, Dallas, Estados Unidos da América), (100 mg/Kg, 100 µL s.c. ou 100 µg/pata, 20 µL, i.pl., pré-tratamento 30 minutos antes do estímulo (doses padronizadas no laboratório em experimentos preliminares). Após o estímulo com a jararagina (1µg/pata) os animais foram avaliados quanto à hiperalgesia mecânica nos tempos 0,5, 1, 2, 3, 4, 5 e 7 horas.

Para avaliar a possível potenciação das citocinas com a jararagina os animais receberam injeção intraplantar de jararagina (0,1 ng/pata), TNF-α (0,1 pg/pata), IL-1β (1 pg/pata) ou co-injeção de jararagina com uma das citocinas. As doses escolhidas induzem pouca ou nenhuma resposta hiperalgésica *per se* (Cunha et al., 2005, dados deste trabalho). A hiperalgesia mecânica foi avaliada 0,5 – 7 h após a administração do estímulo.

3.3.1 Hiperalgesia Mecânica

A avaliação da hiperalgesia mecânica foi realizada pelo método de von Frey (von Frey, 1896), modificado por Cunha et al. (2004), com auxílio de um analgesímetro eletrônico (Modelo 1601C, Life Science Instruments). Esse aparelho consiste em um transdutor de pressão adaptado a um contador digital de força expressa em gramas (g). O contato do transdutor de pressão com a pata é realizado através de uma ponta descartável de polipropileno. Os animais foram colocados em placas de acrílico, constituída por uma rede de arame não maleável, durante 15 minutos antes do experimento para adaptação ao ambiente. Foi realizada uma medição antes da administração do estímulo, tempo zero, e após administração intraplantar da jararagina e nos intervalos de tempo determinados. Para cada tempo, foi considerada a média de três medições. Os resultados foram relatados como delta (Δ) da força (g), sendo calculado subtraindo o valor das medições após estimulo do tempo zero. A intensidade de hiperalgesia foi quantificada como a variação na pressão (Δ de reação em gramas) obtida (Cunha et al., 2004).

3.3.2 Dosagens de TNF-α e IL-1β

A dosagem de citocinas, foi avaliada a quantidade de TNF- α e IL- 1β no tecido plantar dos grupos salina e no grupo com jararagina. As amostras de tecido plantar foram coletadas 1, 3 e 5 horas após o estímulo e no caso do pré- tratamento com PDTC foi coletada na terceira hora após o estimulo com jararagina e homogenizadas (ULTRA-

TURRAX® - Ika) em tampão para dosagem de TNF- α e IL- 1β utilizando kits comerciais de ELISA de acordo com as normas do fabricante (eBioscience, Ready-SET-Go).

3. 4 Análise Estatística

Os resultados obtidos foram expressos como média ± erro padrão da média (EPM) de 6 animais por grupo por experimento. Os experimentos foram realizados em duplicata. A análise de variância two way (ANOVA) foi utilizada para comparar os grupos e doses em todos os tempos quando a hiperalgesia foi medida em tempos diferente após a administração de jararagina. Os fatores analisados foram os tratamentos, tempo e tempo versus tratamento. Quando houve um tempo significativo em relação ao tratamento de interação, analise de variança one way (ANOVA) seguido pelo Tukey's *t*-test foi realizado para cada tempo. Por outro lado, quando as respostas de hiperalgesia foram medidas uma vez após a injeção de estímulo, as diferenças entre as respostas foram avaliadas por ANOVA one way seguido pelo Tukey's t-test. As diferenças estatísticas foram consideradas significativas em $P < 0,05$.

4 RESULTADOS E DISCUSSÃO

Os resultados do atual trabalho estão descritos no artigo científico submetido à revista Toxicon com o título "**Jararhagin-induced mechanical hyperalgesia depends on TNF-α, IL-1β and NFκB in mice**". As normas da revista encontram-se no Anexo A.

4.1 JARARHAGIN-INDUCED MECHANICAL HYPERALGESIA DEPENDS ON TNF-A, IL-1B AND NFKB IN MICE

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Abstract

Jararhagin is a hemorrhagic metalloprotease from *Bothrops jararaca* snake venom. The hyperalgesic mechanisms of jararhagin were investigated focusing on the role of proinflammatory cytokines (TNF-α and IL-1β) and the transcription factor NFκB. Intraplantar administration of jararhagin (1, 10, 100 and 1000 ng/paw) induced mechanical hyperalgesia, and increased TNF-α levels at 1, 3 and 5 h, and IL-1β levels at 0.5, 1 and 3 h after its injection in the paw tissue. Pre-treatment with morphine (2, 6, 12 µg/paw) inhibited jararhagin-induced mechanical hyperagesia. The systemic or local pre-treatment with etanercept (10 mg/Kg and 100 µg/paw) and IL-1ra (30 mg/Kg and 100 pg/paw) inhibited jararhagin-induced mechanical hyperalgesia. Co-administration of jararhagin (0.1 ng/paw) and TNF-α (0.1 pg/paw) or jararhagin (0.1 ng/paw) and IL-1β (1 pg/paw) enhanced the mechanical hyperalgesia. The systemic or local pre-treatment with PDTC (NFκB inhibitor; 100 mg/Kg and 100 µg/paw) inhibited jararhagin-induced mechanical hyperalgesia as well as PDTC decreased the jararhagin-induced production of TNF-α and IL-1β. Thus, these data demonstrate the involvement of pro-inflammatory cytokines TNF-α and IL-1β and nuclear transcription factor NFκB in jararhagin-induced mechanical hyperalgesia indicating that targeting these mechanisms might contribute to reduce the pain induced by *Bothrops jararaca* snake venom.

Keywords

Jararhagin; Metalloprotease; Cytokines; Hyperalgesia; NFκB.

1 Introduction

The venom of *Bothrops jararaca* is a complex mixture of several classes of toxins including serine proteinases, C-type lectins, bradikinin potentiating peptides, phospholipase A2, cysteine-rich proteins, L-amino acid oxidases, and snake venom vascular endothelial growth factor. Of note, the most abundant components of *B. jararaca* are metalloproteases (Cidade et al., 2006, Zelanis et al., 2011). Jararhagin was the first metalloprotease isolated from *B. jararaca* snake venom and its main structure has been characterized (Paine et al., 1992). Many studies showed the involvement of jararhagin in systemic and local damaging effects of snakebite envenoming (Moura-da-Silva and Baldo, 2012). The snake venom metalloproteases (SVMPs) are primarily responsible for the tissue damage and inflammatory responses in *Bothrops* snakebites, including tissue necrosis, hemorrhage and edema (Gutierrez and Rucavado, 2000).

Jararhagin is responsible for many effects of *B. jararaca* snake venom. For instance, jararhagin contributes to the anticoagulant effect by cleaving fibrinogen (Kamiguti et al., 1994), degrading fibrin (Baldo et al., 2008), inhibiting collagen and ristocetin-induced platelet aggregation (Kamiguti et al., 1996), and inhibiting collagen-induced platelet aggregation (Kamiguti et al., 2000). Indeed, the absence of the coagulation process is primarily responsible for the hemorrhagic effect of *B. jararaca* snake venom (Cardoso et al., 1993, Moura-da-Silva and Baldo, 2012).

Jararhagin induces the production of cytokines such as TNF-α and IL-1β *in vivo* (Clissa et al., 2006, Laing et al., 2003), and TNF-α and IL-1β mRNA expression *in vitro* (Clissa et al., 2001). The tumor necrosis factor-alpha (TNF-α) plays a key role in triggering inflammatory pain (Cunha et al., 1992). Varied inflammatory stimuli such as lipopolysaccharide (LPS) and carrageenan induce the release of bradykinin which stimulates the release of TNF-α. TNF-α induces the production of IL-6 and IL-1β, which in turn stimulate the activation of cyclooxygenase resulting in prostanoid production and inflammatory hyperalgesia. TNF-α is also capable of inducing the release of chemokines such as CXCL1, which stimulate the release/production of sympathetic amines (Cunha et al., 1991, Cunha et al., 1992, Ferreira et al., 1988, Ferreira et al., 1993a, Ferreira et al., 1993b, Lorenzetti et al., 2002).

The transcription factor NFκB regulates cytokine production. In fact, inhibiting NFκB activation with drugs such as PDTC (pyrrolidine dithiocarbamate) reduces cytokine production in varied models of inflammation (Ivan et al., 2014, Schreck et

al., 1992). In agreement with the role of cytokines in pain, inhibiting NFκB activation also reduces pain (Possebon et al., 2014, Tegeder et al., 2004). Pain is an important clinical component of snakebites (Bonavita et al., 2006, Rocha et al., 2000, Zychar et al., 2010) and the mechanisms underlying *B. jararaca* snake venom and jararhagin-induced pain are incompletely understood. Despite jararhagin induction of TNF-α and IL-1β production in other systems (Clissa et al., 2006, Laing et al., 2003), and the hyperalgesic role of TNF-α and IL-1β, and NFκB (Verri et al., 2006), the contribution of cytokines and NFκB to jararhagin-induced mechanical hyperalgesia remains to be determined.

2 Material and methods

2.1. Animals

The experiments were performed on male Swiss mice (20–25 g, Universidade Estadual de Londrina, Londrina, PR, Brazil) housed in standard clear plastic cages with free access to food and water. All behavioral testing was performed between 9:00 am and 5:00 pm in a temperature-controlled room. Animals' care and handling procedures were in accordance with the International Association for Study of Pain (IASP) guidelines and with the approval of the Ethics Committee of the Universidade Estadual de Londrina (CEUA Nº 7786.2014.42).

2.2 Jararhagin purification

The jararhagin was purified as previously described methodology (Moura-da-Silva et al., 2003, Paine et al., 1992) in the Laboratory of Immunopathology, Butantan Institute. Jararhagin was subjected to treatment with Triton X-114 as described by Aida and Pabst (1990) to remove any possible contamination with LPS and the presence of LPS was disproved by the LAL test (Limuls Amebocyte Lysate).

2.3 Drugs

Drugs used were: morphine (2, 6 or 12 µg/paw, 20 µL, 30 min before stimulus) from Cristalia (São Paulo, Brazil); etanercept (10 mg/Kg, 200 µL, i.p., 48 h plus 1 h before stimulus or 100 µg/paw, 20 µL, 1 h before stimulus) from Wyeth Indústria Farmacêutica Ltda (São Paulo, Brazil); interleukin-1 receptor antagonist (IL-1ra) (30 mg/Kg, 200 µL, i.p. or 100 pg/paw, 20 µL, i.pl., 30 min before stimulus) from NIBSC (National Institute of Biological Standards and Control, UK): pyrrolidine dithiocarbamate (PDTC) (100 mg/Kg, 100 µL s.c. or 100 µg/paw, 20 µL, i.pl., 30 min before stimulus) from Santa Cruz Biotechnology (Dallas, United States); TNF-α (0,1 pg/paw, 20 µL, i.pl., concomitantly with jararhagin) from eBioscience and IL-1β (0,1 pg/paw, 20 µL, i.pl., concomitantly with jararhagin) from eBioscience.

2.4 Experimental protocols

Mice received intraplantar (i.pl.) injection jararhagin (1, 10, 100, 1000 ng/paw) and mechanical hyperalgesia was evaluated after 0.5, 1, 2, 3, 4, 5, 7 h. Regarding the effect of pharmacological treatments over mechanical hyperalgesia mice were

treated with morphine (2, 6 or 12 µg/paw, 20 µL, i.pl., 30 min before stimulus), etanercept (10 mg/Kg, 200 µL, i.p., 48 h and 1 h before stimulus or 100 µg/paw, 20 µL, i.pl., 1 h before stimulus), IL-1ra (30 mg/Kg, 200 µL, i.p. or 100 pg/paw, 20 µL, i.pl., 30 min before stimulus), PDTC (100 mg/Kg, 100 µL s.c. or 100 µg/paw, 20 µL, i.pl., 30 min before stimulus) followed by intraplantar (i.pl.) administration of jararhagin (1 µg/paw) and mechanical hyperalgesia was evaluated after 0.5 - 7 h. In other settings, mice received jararhagin (0.1 ng/paw, i.pl), TNF-α (0.1 pg/paw, i.pl.) or IL-1β (1 pg/paw, i.pl.) injection separately or the co-injection of jararhagin and TNF-α or jararhagin and IL-1β and mechanical hyperalgesia was evaluated between 0.5 - 7 h. Cytokine (TNF-α and IL-1β) levels were determined 0.5, 1, 3 and 5 h after jararhagin injection (1 µg/paw) or at 3 h after jararhagin injection (1 µg/paw) in mice pre-treated with PDTC (100 mg/Kg, 100 µL s.c. 30 min before stimulus). Doses of treatment were based on previous studies of our group and standardization in our laboratory (Borghi et al., 2014a, Borghi et al., 2014b, Carvalho et al., 2015, Carvalho et al., 2011, Ivan et al., 2014).

2.5 Electronic pressure–meter test for mice

Mechanical hyperalgesia was tested in mice as previously reported (Cunha et al., 2004). Briefly, the test consists of evoking a hindpaw flexion reflex with a hand-held force transducer (the electronic von Frey anesthesiometer: Insight, Ribeirão Preto, SP, Brazil) adapted with a 0.5 mm^2 contact area polypropylene tip. The investigator was trained to apply the tip perpendicularly to the central area of the hindpaw and the endpoint was characterized by the removal of the paw. The results are expressed by delta (Δ) withdrawal threshold (in g), which was calculated by subtracting the zero-time mean measurements from the mean measurements (indicated time points) after stimulus.

2.6 Cytokine Measurement

Plant tissue samples were collected at indicated time points after stimulus with jararhagin, and homogenized using a ultraturrax (ULTRA-TURRAX® - Ika) in buffer containing protease inhibitors for measurement of TNF-α and IL-1β levels using commercial ELISA kits according to the manufacturer's instructions (eBioscience®, Ready-SET-Go).

2.7 Statistical Analysis

Results are presented as means ± S.E.M. of measurements made on 6 mice per group per experiment. The experiments were performed twice. Two-way analysis of variance (ANOVA) was used to compare the groups and doses at all times (curves) when the hyperalgesic responses were measured at different times after the stimulus injection. The analyzed factors were treatments, time and time *versus* treatment interaction. When there was a significant time *versus* treatment interaction, one-way ANOVA followed by Tukey's *t*-test was performed for each time. On the other hand, when the hyperalgesic responses were measured once after the stimulus injection, the differences between responses were evaluated by one-way ANOVA followed by Tukey's t-test. Statistical differences were considered to be significant at $P < 0.05$.

3 Results

3.1 Jararhagin induces dose-dependent mechanical hyperalgesia

Mice received 1, 10, 100 or 1000 ng of jararhagin by intraplantar route (ipl; subcutaneous injection in the paw). Jararhagin was diluted in saline (20 µl), which was used as vehicle control group. Mechanical hyperalgesia was evaluated 0.5, 1, 2, 3, 4, 5 and 7 hours after the injection of Jararhagin (Figure 1). Jararhagin induced dose-dependent mechanical hyperalgesia. The mechanical hyperalgesia was significant compared to saline control group up to 3 h for jararhagin at 1 and 10 ng, up to 5 h for jararhagin at 100 ng and up to 7 h for jararhagin at 1000 ng. The hyperalgesic response of 1000 ng of jararhagin was significantly higher than the lower doses, thus, it was selected for the next experiments except by figure 6. **(Include Figure 1 here).**

3.2 Jararhagin-induced response was amenable by morphine confirming its nociceptive characteristic.

Mice were treated with morphine (opioid receptor agonist; at 2, 6 and 12 µg/paw) 0.5 hours before the stimulus with jararhagin (1 ug/paw) or saline (20 µl/paw), and mechanical hyperalgesia was evaluated at 0.5 h after stimulus injection considering this is the peak of jararhagin hyperalgesia and the effect of morphine at these doses is transient and lasts for approximately 1-2h (Verri et al., 2004) (Figure 2). Morphine dose-dependently inhibited jararhagin-induced mechanical hyperalgesia at the doses of 2 and 6 µg/paw and the dose of 12 µg/paw presented statistically significant inhibition compared to the lower dose of morphine tested (Figure 2). Therefore, jararhagin-induced reduction of mechanical threshold is amenable by morphine treatment. **(Include Figure 2 here).**

3.3 Jararhagin induces time-dependent cytokine production.

Mice received jararhagin (1 µg) or saline i.pl. injection and after 0.5, 1, 3 and 5 h samples were collected and cytokine levels determined by ELISA (Figure 3). Jararhagin induced significantly TNF-α production at 1, 3 and 5 h after its injection (Figure 3A) and IL-1β production at 0.5, 1 and 3 h (Figure 3B). **(Include Figure 3 here).**

3.4 Systemically targeting TNF-α and IL-1β reduce jararhagin-induced mechanical hyperalgesia

Mice were treated with etanercept (soluble tumor necrosis factor receptor II; 10 mg/Kg, i.p.) 48 h and 1 h (Figure 4A) or with IL-1ra (interleukin-1 receptor antagonist; 30 mg/Kg, i.p.) 30 min (Figure 4B) before jararhagin (1 µg/paw) injection. Mechanical hyperalgesia was evaluated between 0.5 and 7 h after stimulus injection (Figure 4). Etanercept and IL-1ra inhibited jararhagin-induced mechanical hyperalgesia at all time points evaluated indicating the role of TNF-α and IL-1β in this nociceptive response (Figure 4). **(Include Figure 4 here).**

3.5 Locally targeting TNF-α and IL-1β reduce jararhagin-induced mechanical hyperalgesia

Mice were treated with etanercept (100 µg/paw, i.pl.) (Figure 5A) or with IL-1ra (100 pg/paw, i.pl.) (Figure 5B) 30 min before jararhagin (1 µg/paw) injection (Figure 5). Mechanical hyperalgesia was evaluated between 0.5 and 7 h after stimulus injection (Figure 5). Etanercept and IL-1ra inhibited jararhagin-induced mechanical hyperalgesia at all time points evaluated (Figure 5). The treatment with etanercept (Figure 5A) or IL-1ra (Figure 5B) in the contra-lateral (CL) paw to jararhagin stimulus did not affect the nociceptive response, which confirms the locally acting effect of etanercept and IL-1ra at these doses, and indicates the role of peripheral TNF-α and IL-1β in jararhagin-induced mechanical hyperalgesia. **(Include Figure 5 here).**

3.6 TNF-α and IL-1β enhance jararhagin-induced mechanical hyperalgesia

Mice received jararhagin (0.1 ng/paw, i.pl), TNF-α (0.1 pg/paw, i.pl.) or IL-1β (1 pg/paw, i.pl.) injection separately or the co-injection of jararhagin and TNF-α or jararhagin and IL-1β. Mechanical hyperalgesia was evaluated between 0.5 - 7 h (Figure 6). TNF-α and jararhagin induced significant mechanical hyperalgesia when administrated separately (Figure 6A), and the co-injection of both resulted in increased nociceptive response with significant statistical differences comparing the co-injection with the separately injections of jararhagin and TNF-α (Figure 6A). It was noticeable at 3, 4, 5 and 7 h that the mechanical hyperalgesia resulting from the co-injection of jararhagin and TNF-α was higher than the sum of the hyperalgesia of separate molecules. Furthermore, while the jararhagin- and TNF-α-induced hyperalgesia vanished by 2 and 5 h, respectively, the hyperalgesia induced by the

co-injection of both molecules was still significant at 7 h (Figure 6A). Separate injection of jararhagin and IL-1β induced mechanical hyperalgesia up to 2 and 4 h, respectively. The co-injection of jararhagin and IL-1β induced mechanical hyperalgesia up to 7 h and with a higher nociceptive response with the combined stimuli than the solely stimulus (Figure 6B) similarly to the response observed with TNF-α (Figure 6A). These data demonstrate that the combined action of jararhagin with the pro-inflammatory molecules that it induces produce an enhanced hyperalgesic response that might contribute to extend and increase the pain sensation. **(Include Figure 6 here).**

3.7 The NFκB inhibitor, PDTC, diminishes jararhagin-induced mechanical hyperalgesia, and TNF-α and IL-1β production.

Mice were treated with PDTC by s.c. (100 mg/Kg; Figure 7A, C and D) or i.pl. (100 µg/paw; Figure 7B) routes 30 min before administration of jararhagin (1 µg/paw). Mechanical hyperalgesia was evaluated between 0.5 and 7 h after stimulus injection (Figure 7A and B) and samples of plantar skin were collected 3 h after jararhagin injection (Figure 7C and D). PDTC treatment inhibited jararhagin-induced mechanical hyperalgesia at all time points evaluated at systemic (Figure 7A) and locally acting (Figure 7B) doses. Mice also received PDTC treatment in the contra-lateral paw to jararhagin stimulus, which did not affect the nociceptive response demonstrating the local action of PDTC at 100 µg/paw (Figure 7B). PDTC also inhibited TNF-α (Figure 7C) and IL-1β (Figure 7D) production. **(Include Figure 7 here).**

4 Discussion

Tissue damage and necrosis, inflammatory response, hemorrhage, edema and pain are common symptoms of snake bite accidents (Dale et al., 2004, Gutierrez and Rucavado, 2000). Among the symptoms of snake bites, pain is less study, although it is clinically relevant to the patient. The present data demonstrate that the metalloprotease of *Bothrops jararaca*, jararhagin, is capable of inducing mechanical hyperalgesia dependent on pro-hyperalgesia cytokines TNF-α and IL-1β. In agreement with this finding, targeting these cytokines with etanercept and IL-1ra or with the NFκB inhibitor PDTC diminish jararhagin-induced mechanical hyperalgesia.

The intraplantar injection of jararhagin induced mechanical hyperalgesia in a dose-dependent manner in mice. Similar data was demonstrated by Dale et al. (2004), which showed that increasing doses of jararhagin induced hyperalgesia in rats. Our finding differ from Dale et al. (2004) by the animal specie (mouse *versus* rat), persistence of mechanical hyperalgesia (7 *versus* 3 h), dose of jararhagin (up to 1 µg *versus* 5 µg), and method of evaluation (electronic version of von Frey *versus* Randall & Selitto method), respectively. All together, these differences might have contributed to the different outcomes of dose and duration of hyperalgesia.

Although it seems obvious that pain can be inhibited by analgesics, not all types of pain are amenable by all analgesics explaining the need of research and advance in this field. Opioids such as morphine do not inhibit all types of pain, but are effective in inflammatory conditions for sure (Cunha et al., 2010, Cunha et al., 2012, Verri et al., 2004). In the present study, jararhagin-induced mechanical hyperalgesia was inhibited by morphine treatment. This result is important in the sense that it was previously undetermined whether the altered mechanical threshold induced by jararhagin (Dale et al., 2004 and present data) administration would be amenable by an analgesic, which is a proof-of-concept of the nociceptive characteristic of jararhagin.

Jararhagin hydrolyzes recombinant pro-TNF-α generating biologically active TNF-α, indicating that this metalloprotease presents a TNF-α convertase activity (Moura-Da-Silva et al., 1996). In agreement with a role for the mediators induced by jararhagin to its effects, jararhagin-induced dermal necrosis was abolished in IL-6, TNF receptor 1 and 2 deficient mice as well as jararhagin induced fast elevation of TNF-α, IL-1β and IL-6 levels in the exposed tissues (Laing et al., 2003). Consistent with these experimental findings, snake bit patients present increased levels of

cytokines in the blood (Barraviera, et al., 1995). These data support our results that jararhagin-induced mechanical hyperalgesia was inhibited by targeting TNF-α and IL-1β systemically and locally, jararhagin induced the production of TNF-α and IL-1β in the paw skin and co-administration of jararhagin with TNF-α or IL-1β potentiated the hyperalgesic actions of this metalloprotease.

TNF-α and IL-1β were the first cytokines described to be hyperalgesic (Ferreira et al., 1988; Cunha et al., 1992). These cytokines are consistent mediators of pain in varied models of inflammation, cancer and neuropathic pain (Borghi et al., 2013, Borghi et al., 2014a, Borghi et al., 2014b, Cunha et al., 2000, Schafers et al., 2001, Verri et al., 2010, Zarpelon et al., 2013, Zelenka et al., 2005). Furthermore, TNF-α and IL-1β have pronounced role in inflammatory disease development, which is consistent with the effectiveness of targeting TNF-α and IL-1β to control, for instance, rheumatoid arthritis disease progression (Verri et al., 2010). In this sense, the effectiveness of etanercept and IL-1ra (endogenous IL-1ra differs from clinically used molecule [anakinra] by 1 aminoacid) in inhibiting jararhagin-induced hyperalgesia support the rationale that targeting these cytokines with clinically used approaches could amenable pain in *B. jararaca* bitten patients. Importantly, considering TNF-α and IL-1β can be activated (Moura-da-Silva et al., 1996) and produced upon jararhagin injection (Moura-da-Silva et al., 1996 and present data), and hence potentiate jararhagin-induced mechanical hyperalgesia (present data), targeting these cytokines seem to have great potential as therapeutic strategies to control snake bite-induced pain.

Besides TNF-α convertase activity (Moura-da-Silva et al., 1996), jararhagin also induces the mRNA expression of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β in culture of mouse peritoneal macrophages (Clissa et al., 2001), which suggests jararhagin would induce the activation of transcription factors that regulate the expression of those molecules. Corroborating this finding, jararhagin-induced hyperalgesia was reduced by local and systemic treatment with PDTC, an inhibitor of NFκB activation. In fact, PDTC treatment inhibited jararhagin-induced TNF-α and IL-1β production.

In addition to the inhibitory effect of PDTC over NFκB activation, it has been reported that PDTC presents antioxidant effect (Ivan et al., 2014). Evidence support that the antioxidant effect of PDTC explains its inhibitory action over NFκB (Schreck et al., 1992) as well as PDTC presents antioxidant effects without inhibiting NFκB

activation (Nathens et al., 1997). Inhibitory mechanisms of PDTC over NFκB not related to antioxidant activity include the interference with κB-dependent transactivation genes (Schreck et al., 1992), ability to translocate extracellular Zn^{2+} to intracellular sites (Kim et al., 1999a, Lee et al., 2008), which has as consequence the inhibition of NFκB activation (Bruck et al., 2002, Kim et al., 1999b), and inhibition of IκB–ubiquitin ligase activity in cell-free system without extracellular stimuli-regulated production of reactive oxygen species (Hayakawa et al., 2003).

The hyperalgesic dose of jararhagin (1 µg/paw) did not reduce the ferric reducing ability potential, the ability to scavenge the radical ABTS, and did not reduce the levels of reduced gluthathione between 0.5-7 h after its injection (data not shown), which indicate a minor role for oxidative stress in jararhagin-induced mechanical hyperalgesia. Thus, PDTC could not be acting by reducing oxidative stress to inhibit NFκB activation since jararhagin did not induce oxidative stress at the hyperalgesic dose of 1 µg/paw. Therefore, it is likely that PDTC inhibition of jararhagin-induced hyperalgesia was related to targeting NFκB activation independently of antioxidant actions.

5 Conclusion

Jararhagin induces mechanical hyperalgesia dependent on pro-inflammatory cytokines TNF-α and IL-1β as well as these cytokines potentiate jararhagin nociception. Therefore, targeting these cytokines with etanercept and IL-1ra or their production with the NFκB inhibitor PDTC inhibits jararhagin-induced mechanical hyperalgesia. These immunobiological and pharmacological approaches could be useful to limit *Bothrops jararaca*-induced pain, which merits further pre-clinical and clinical investigation.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Figure Captions

Figure 1. Jararhagin induces dose-dependent mechanical hyperalgesia. Mice received jararhagin via i.pl. 1, 10, 100, 1000 ng/paw or saline (20µL). Mechanical hyperalgesia was evaluated after 0.5 - 7 h. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. *P<0.05 compared with saline group, #P<0.05 compared with lower doses and saline group, fP<0.05 compared with 1, 10 ng and saline group. ANOVA followed by Tukey's t-test.

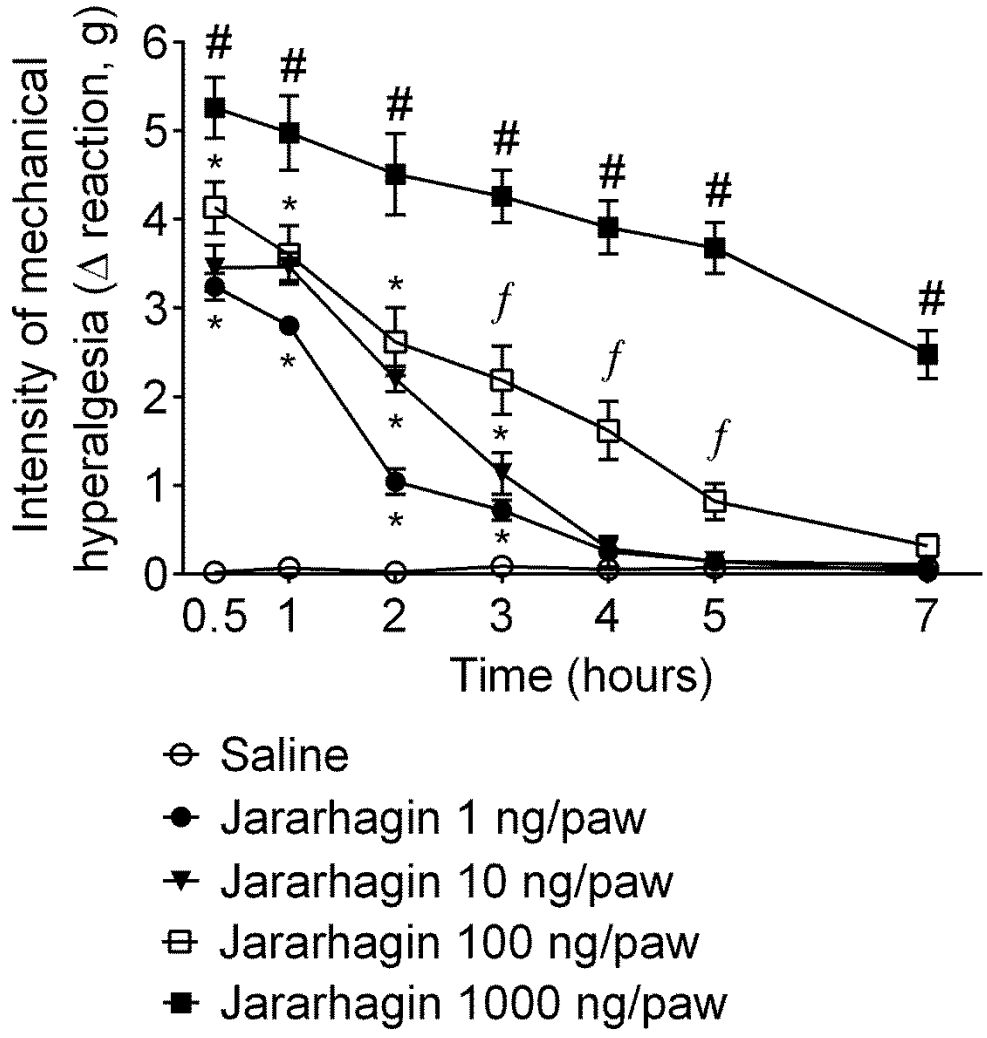


Figure 2. Morphine inhibited jararhagin-induced mechanical hyperalgesia in a dose-dependent manner. Mice were treated with morphine (2, 6, 12 µg/paw) or vehicle 0.5 hour before i.pl. injection of jararhagin (1 µg/paw, 20 µL, i.pl.). Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. *P<0.05 compared with saline group, #P<0.05 compared with jararhagin group and **P<0.05 compared with lower doses of morphine. ANOVA followed by Tukey's t-test.

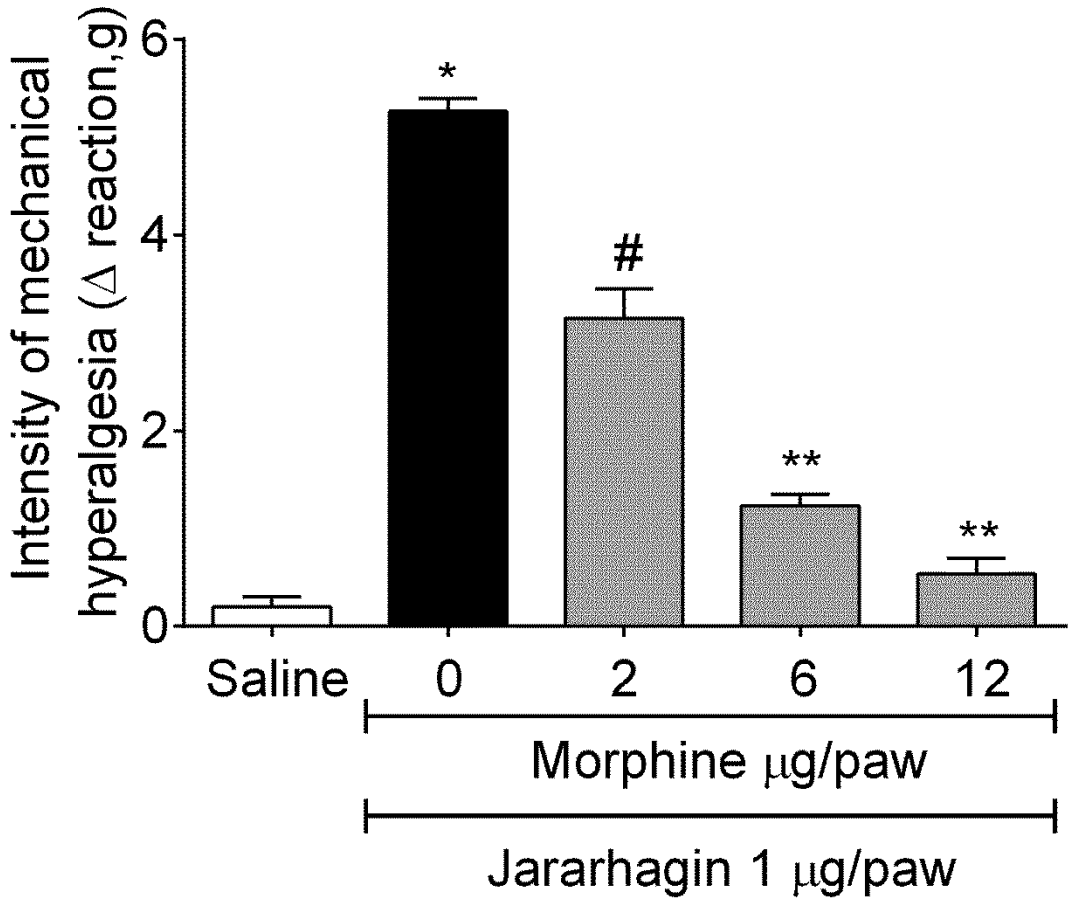


Figure 3. Jararhagin induces TNF-α (A) and IL-1β (B) production in the mice paw skin. Mice received i.pl. injection of jararhagin (1 µg/paw) or saline (20 µL). Paw skin samples were collected at 0.5, 1, 3 and 5 hour after stimulus with jararhagin, and TNF-α and IL-1β levels measured by ELISA. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. *P <0.05 compared with saline group, #P <0.05 compared with saline and 0.5 h group *#P<0.05 compared with saline, 0.5 hour and 5 hour group. ANOVA followed by Tukey's t-test.

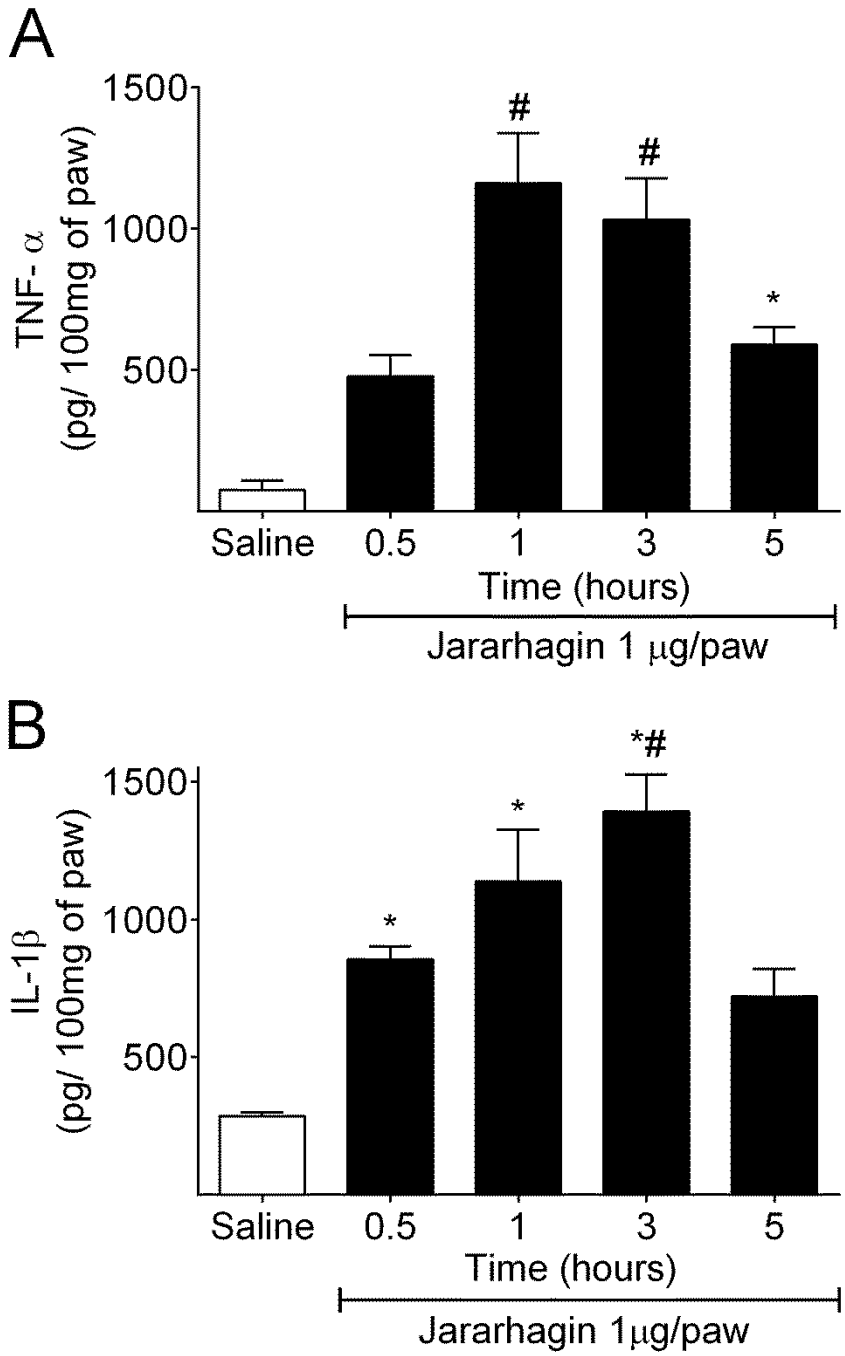


Figure 4. Systemic treatment with etanercept and IL-1ra reduced jararhagin-induced mechanical hyperalgesia in mice. Mice were pre-treated with etanercept (10 mg/Kg, 200 µL i.p., 48 h and 1 h) (Panel A), IL- 1ra (30 mg/Kg, 200 µL i.p.) (Panel B) or equivalent volume of saline before i.pl. injection of jararhagin (1 µg/paw, 20 µL). Mechanical hyperalgesia was evaluated after 0.5 - 7 h. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. *P<0.05 compared with saline group and #P<0.05 compared with jararhagin group. ANOVA followed by Tukey's t-test.

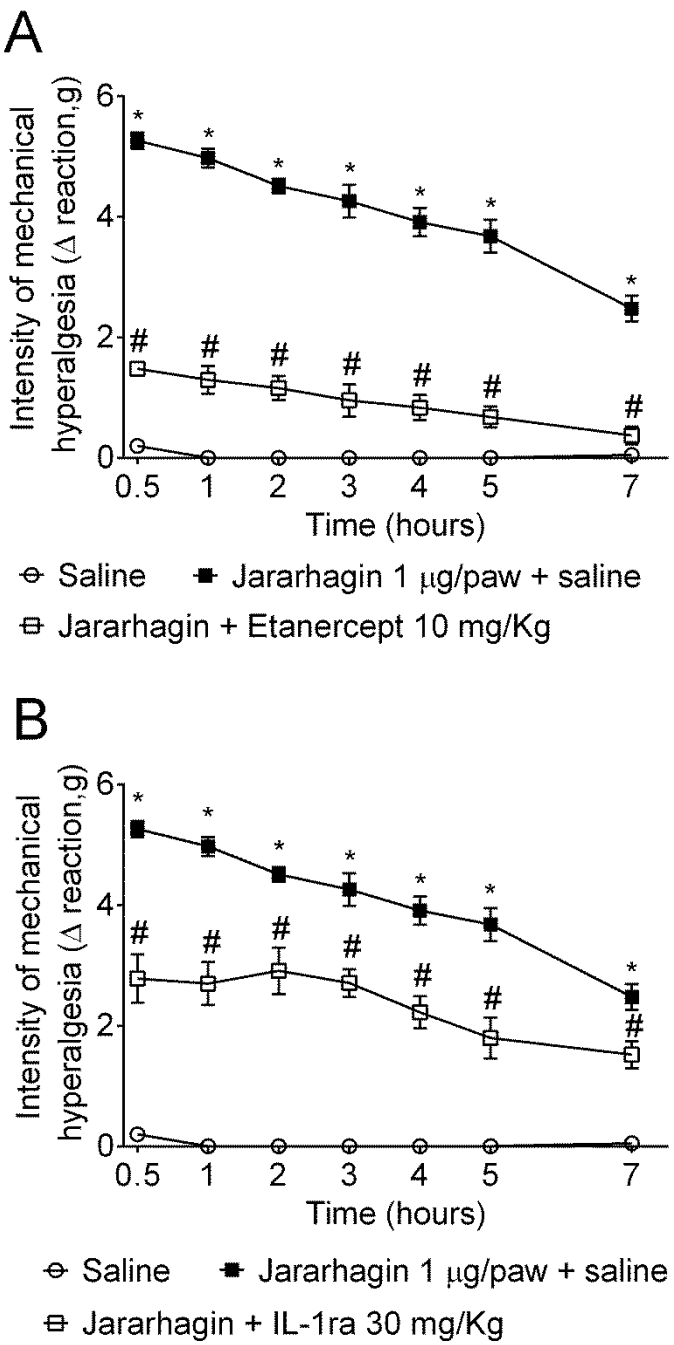
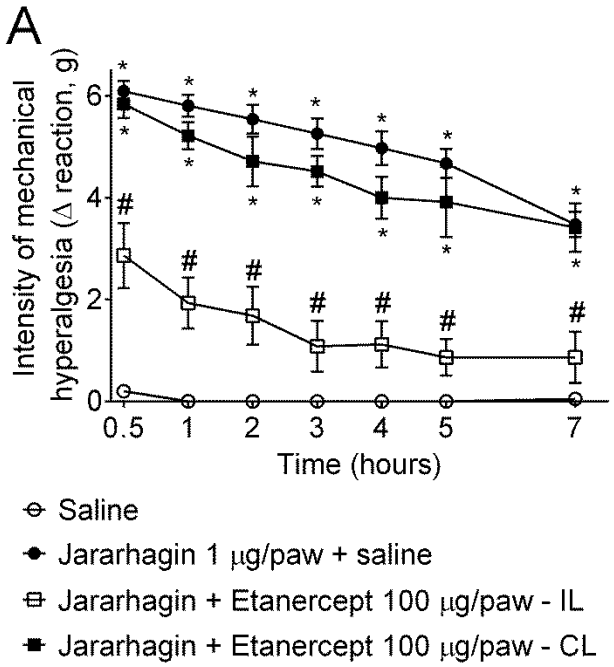


Figure 5. Local treatment with etanercept and IL-1ra reduced jararhagin-induced mechanical hyperalgesia in mice. Mice were treated with etanercept (100 µg/paw, i.pl.) (Panel A), IL-1ra (100 pg/paw, i.pl.) (Panel B) 1 h or equivalent volume of saline before jararhagin (1 µg/paw) injection. Mechanical hyperalgesia was evaluated between 0.5 - 7 h after stimulus injection, and mechanical hyperalgesia was evaluated in the ipsilateral and contra-lateral paw to the stimulus. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. *P<0.05 compared with saline group and #P<0.05 compared with jararhagin group. ANOVA followed by Tukey's t-test.



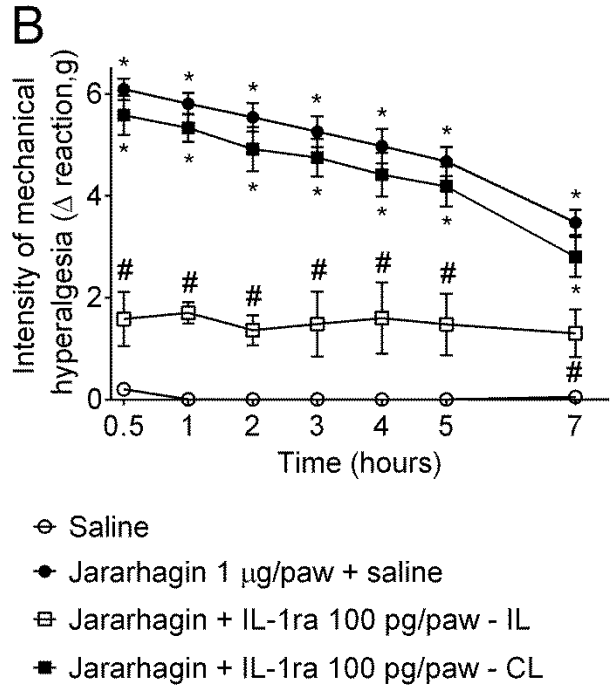
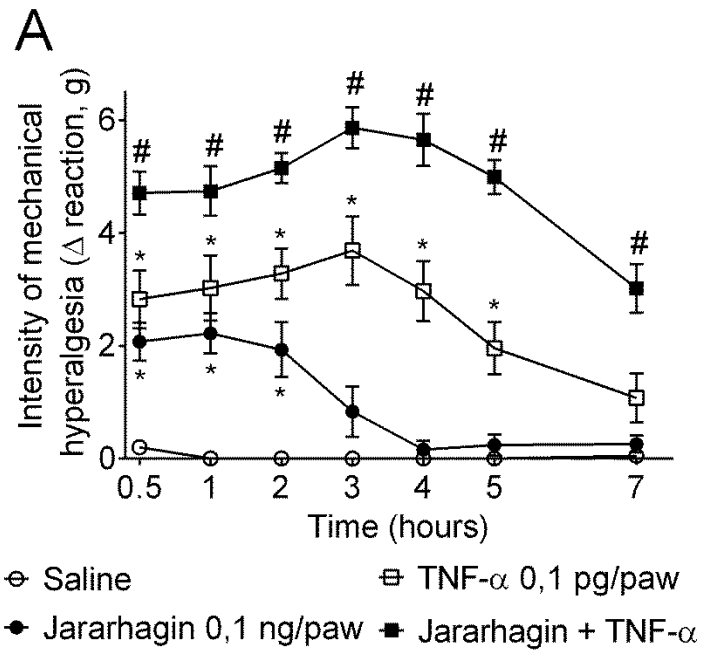


Figure 6. TNF-α and IL-1β increase jararhagin-induced mechanical hyperalgesia. Mice received jararhagin (0.1 ng/paw, i.pl), TNF-α (0.1 pg/paw, i.pl.) or IL-1β (1 pg/paw, i.pl.) injection separately or the co-injection of jararhagin and TNF-α (Panel A) or jararhagin and IL-1β (Panel B). Mechanical hyperalgesia was evaluated between 0.5 - 7 h after stimulus injection. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. *P<0.05 compared with saline group and #P<0.05 compared with TNF-α or IL-1β and jararhagin group. ANOVA followed by Tukey's t-test.



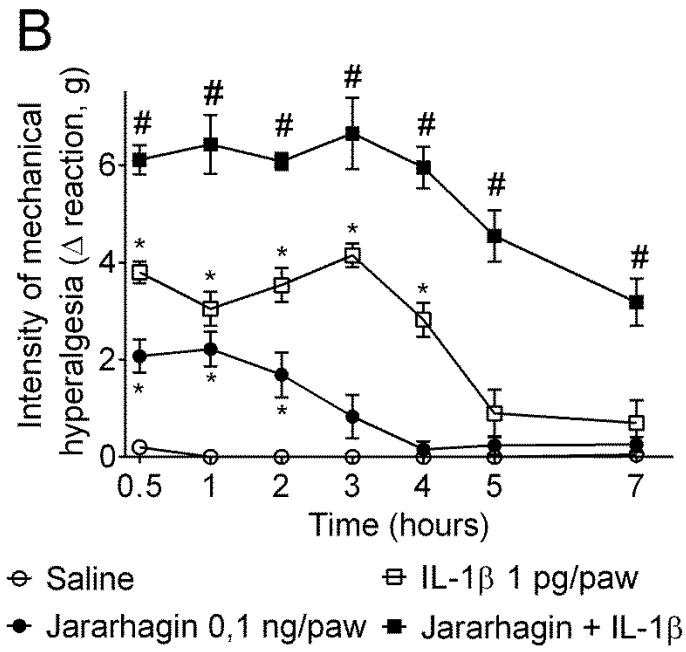
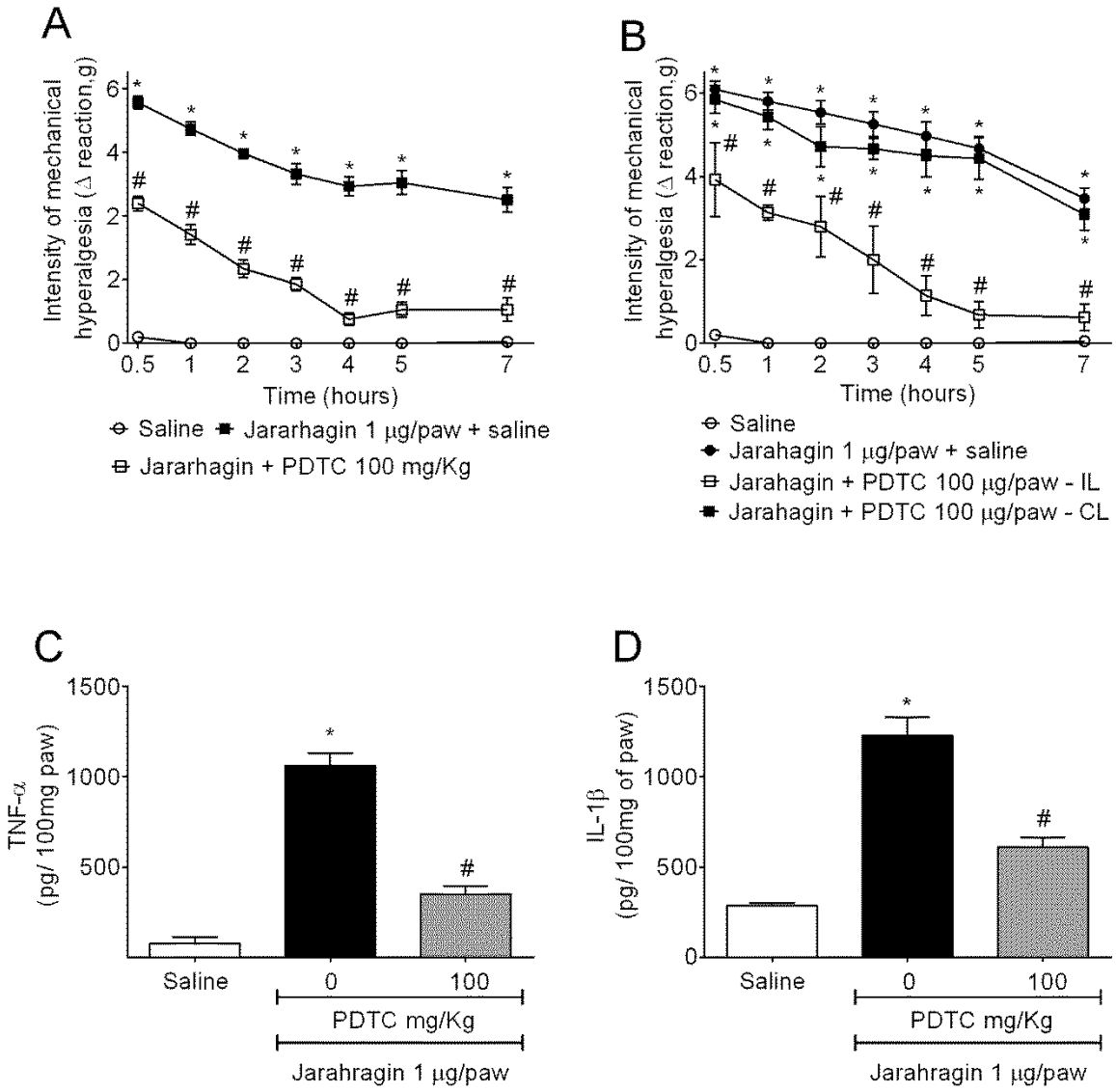


Figure 7. Treatment with PDTC reduced jararhagin-induced mechanical hyperalgesia and TNF-α and IL-1β production. Mice were treated with PDTC by s.c. (100 mg/Kg) (Panel A) or i.pl. (100 µg/paw) (Panel B) routes 30 min before administration of jararhagin (1 µg/paw). Mechanical hyperalgesia was evaluated between 0.5 - 7 h after stimulus injection (Panel A and B), samples of plantar skin were collected 3 h after jararhagin injection (Panel C and D), and TNF-α (Panel C) and IL-1β (Panel D) levels measured by ELISA. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. *P<0.05 compared with saline group and #P<0.05 compared with jararhagin. ANOVA followed by Tukey's t-test.



5 CONCLUSÃO

Portanto, a jararagina induz hiperalgesia mecânica dependente das citocinas pró-inflamatórias TNF-α e IL-1β e que as mesmas potencializam a nocicepção induzida pela jararagina. Assim, tendo essas citocinas como alvo, a hiperalgesia induzida pela jararagina foi inibida pelo pré tratamento com etanercept (receptor solúvel de TNF-α) e IL-1ra (antagonista de IL-1) ou PDTC (inibidor do NFκB). Essas abordagens farmacológicas e imunobiológicas podem ser úteis para diminuir a dor induzida pelo veneno da *Bothrops jararaca*, a qual necessita de maiores investigações tanto pré-clínicas quanto clínicas.

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ANEXOS

ANEXO A

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Official Journal of The International Society on Toxinology (http://www.toxinology.org/), *Toxicon*'s "aims and scope" are laid down in the journal as:

To publish:

- articles containing the results of original research on problems related to toxins derived from animals, plants and microorganisms
- papers on novel findings related to the chemical, pharmacological, toxicological, and immunological properties of natural toxins
- molecular biological studies of toxin and other genes from poisonous and venomous organisms that advance understanding of the role or function of toxins
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And
To encourage the exchange of ideas, sections of the journal may be devoted to Short Communications, Letters to the Editor and activities of the International Society on Toxinology.

Toxicon strives to publish articles that are current and of broad interest and importance to the toxinology research community. Emphasis will be placed upon articles that further the understanding and knowledge of toxinology.

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