Universidade Estadual de Londrina

CAMILA RODRIGUES FERRAZ

ESTUDO EM CAMUNDONGOS DE MECANISMOS NOCICEPTIVOS E INFLAMATÓRIOS DE VENENOS DE BOTHROPS sp. E TITYUS ssp.

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Londrina, 22 de julho de 2019.

Àqueles que ainda acreditam na ciência brasileira.

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"Quando a educação não é libertadora, o sonho do oprimido é ser o opressor."

-Paulo Freire

FERRAZ, Camila Rodrigues. **Estudo em camundongos de mecanismos nociceptivos e inflamatórios de venenos de Bothrops sp. e Tityus ssp.** 2019. 121 f. Tese (Doutorado em Ciências da Saúde) – Universidade Estadual de Londrina, Londrina, 2019.

RESUMO

Os acidentes envolvendo cobras e escorpiões apresentam uma grande importância epidemiológica no Brasil e no mundo. A dor é um importante efeito local do envenenamento pela serpente *Bothrops jararaca* e pelos escorpiões *Tityus serrulatus* e *Tityus bahiensis*. Pouco se sabe sobre os mecanismos envolvidos na dor induzida por veneno de serpentes e escorpiões. A jararagina é uma metaloproteinase do veneno de *Bothrops jararaca* capaz de induz hiperalgesia mecânica em modelos murinos. O presente estudo investigou a participação de mecanismos espinais, avaliando o envolvimento das células da glia (astrócitos e micróglia) e das proteínas quinases ativadas por mitógenos (MAPK; ERK, p38 e JNK) na hiperalgesia mecânica induzida por essa metaloproteinase. Para isso, camundongos Swiss foram estimulados com jararagina (1 µg/pata) e a ativação das células da glia, produção de citocinas e a ativação das MAPKs foram avaliadas na medula espinal. A jararagina induziu a ativação dos astrócitos e da micróglia, produção de citocinas pró-inflamatórias (TNF-α e IL-1β) e a fosforilação da p38 e JNK e a na medula espinal. Para verificar a participação das células da glia, das citocinas, do NFkB e da via MAPKs na hiperalgesia induzida pela jararagina, os animais foram tratados por via intratecal com inibidor de TNF-α (etanercept), IL-1β (IL-1ra), NFkB (PDTC), micróglia (minociclina), astrócitos (α-aminoadipate), ERK (PD 98059), p38 (SB 202190) ou JNK (SP 600125) antes do estímulo periférico com a jararagina. Todos os tratamentos testados foram capazes de diminuir a hiperalgesia. Desta maneira, demonstramos a participação de mecanismos espinais na hiperalgesia mecânica induzida pela jararagina, os quais incluem a ativação das células da glia, produção de citocinas pró-inflamatórias na medula espinal via NFkB e ativação das MAPKs espinais. Investigou-se os mecanismos hiperalgésicos induzidos pelos venenos de *Tityus bahiensis* e *Tityus serrulatus*, avaliando o papel do recrutamento de celular, citocinas pró-inflamatórias (TNF-α e IL-1β) e do fator de transcrição NFκB. Para isso, camundongos Swiss foram estimulados com os venenos de *Tityus bahiensis* e *Tityus serrulatus* nas dose de 0,2, 0,6, 1,2 e 2,4µg/20µL i.pl. e a hiperalgesia mecânica e térmica induzida foram avaliadas das 0.5 -5 h após o estímulo e a avaliação da atividade da MPO e NAG foram avaliadas 5h após injeção de veneno de escorpião na pata. A dose 2,4µg dos venenos de *Tityus bahiensis e Tityus serrulatus* induziram dor espontânea e aumentaram os níveis de TNF-α e IL-1β após injeção. O pré-tratamento sistêmico com etanercept (10 mg/kg), IL-1ra (30 mg/kg) e PDTC (inibidor de NFκB; 100 mg/kg) inibiram a hiperalgesia mecânica e térmica induzida pelos venenos de *Tityus bahiensis* e *Tityus serrulatus*, atividade da MPO e NAG e dor espontânea. Assim, esses dados demonstram que o recrutamento de neutrófilos e macrófagos, citocinas pró-inflamatórias, TNF-α e IL-1β, e do fator nuclear de transcrição NFκB desempenham um papel importante na nocicepção induzida pelos venenos de *Tityus bahiensis* e *Tityus serrulatus*. Dessa maneira, a compreensão dos fatores moduladores na dor induzida por venenos de serpentes e escorpiões são essenciais para o

desenvolvimento de novas estratégias terapêuticas mais eficazes e propiciem uma assistência de melhor qualidade às vítimas de acidentes ofídicos e escorpiônicos.

Palavras-chave: Veneno de serpentes. Veneno de escorpião. Citocinas. Dor. Microglia. Astrócitos. MAP quinases.

FERRAZ, Camila Rodrigues. **Study of nociceptive and inflammatory mechanisms of Bothrops sp. and Tityus ssp. venoms.** 2019. 121 p. Thesis (Doctorate degree in Health Sciences) – Universidade Estadual de Londrina, Londrina, 2019.

ABSTRACT

Snake and scorpion envenomation cause a significant health and economic burden in Brazil and in the worldwide. *Bothrops jararaca* bite and *Tityus bahiensis* and *Tityus serrulatus* sting cause severe local pain. Very little is known about the pain pathways induced by snake and scorpion venoms. Jararhagin, a metalloprotease from *Bothrops jararaca* venom, is able to induce mechanical hyperalgesia in animal model. Herein, we investigated the involvement of glial cells (astrocytes and microglia) and mitogen-activated protein kinase (MAPK; ERK, p38 and JNK) in mechanical hyperalgesia induced by jararhagin. We evaluated the activation of glia cells, cytokines production and activation of MAPKs in spinal cord following injection of jararhagin (1 µg/paw) in Swiss mice. Jararhagin induced astrocytes and microglia activation, TNF-α, and IL-1β production and p38 and JNK phosphorylation. Inhibitor of TNF-α (etanercept), IL-1β (IL-1ra), NFkB (PDTC), microglia (minocycline), astrocyte (α-aminoadipate), ERK (PD 98059), p38 (SB 202190) or JNK (SP 600125) were intrathecally injected in mice before stimulus with jararhagin to evaluate the involvement of glia cells, cytokines, NFkB and MAPKs pathway in mechanical hyperalgesia induced by this metalloprotease. All inhibitors reduced jararhagin-induced hyperalgesia. Thus, we demonstrated the spinal mechanisms in jararhagin-induced hyperalgesia which involves the activation of spinal cells, NFκB-induced production of pro-inflammatory cytokines and activation of MAPK pathway in the spinal cord. Finally, the hyperalgesic mechanisms of *Tityus bahiensis* and *Tityus serrulatus* venom were investigated focusing on the role of recruitment of cells, proinflammatory cytokines (TNF-α and IL-1β) and the transcription factor NFκB. Intraplantar administration of *Tityus bahiensis* and *Tityus serrulatus* venom 0.2, 0.6, 1.2 and 2.4µg/20 µL i.pl. induced mechanical and thermal hyperalgesia, and increased MPO and NAG activity at 5h after scorpion venoms injection in the paw tissue. *Tityus bahiensis* and *Tityus serrulatus* venom (2.4 µg) induced overt pain-like behaviour and increased TNF-α levels at and IL-1β levels after scorpion venoms injection in the paw tissue. The systemic pre-treatment with etanercept (10 mg/kg), IL-1ra (30 mg/kg) and PDTC (NFκB inhibitor; 100 mg/kg) inhibited *Tityus bahiensis* and *Tityus serrulatus* venom-induced mechanical and thermal hyperalgesia, MPO and NAG activity and overt pain-like behaviour. Thus, these data demonstrate the involvement of neutrophils, macrophages, pro-inflammatory cytokines TNF-α and IL-1β and nuclear transcription factor NFκB in *Tityus bahiensis* and *Tityus serrulatus* venom-induced mechanical thermal hyperalgesia and overt pain-like behaviour indicating that targeting these mechanisms might contribute to reduce the pain induced by scorpion venoms. Understanding the mechanisms activated by snake and scorpion venoms are essential to develop new effective treatments and enables a better management of snakebite and scorpionism patients.

Keywords: Snake venom. Scorpion venom. Cytokines. Pain. Microglia. Astrocytes. MAP kinases.

SUMÁRIO

1 INTRODUÇÃO

Estudos farmacológicos, genéticos e moleculares têm avançado na compreensão dos mecanismos envolvidos na transmissão do sinal doloroso em modelos experimentais de doença [1-9].

O envenenamento por animais peçonhentos representam um grande ônus para a saúde pública em todo o mundo [10]. Várias espécies de animais têm o potencial de causar envenenamento em seres humanos, mas as cobras e os escorpiões apresentam grande importância epidemiológica no Brasil e no mundo [11, 12].

A picada de animais peçonhentos, como cobras e escorpiões e outros, compartilha alguns sinais e sintomas; dentre eles, a dor manifesta-se comumente nesses acidentes [13-15].

1.1 Acidente ofídicos

Em todo o mundo, até 5 milhões de pessoas são picadas por cobras todos os anos, o que causam considerável morbilidade e mortalidade. Estima-se, que pelo menos, 1,8 milhões envenenamentos e 81 mil mortes ocorrem a cada ano devido à picada de cobra em todo o mundo [11, 16].

Em 2017, a Organização Mundial da Saúde (OMS) reconheceu o acidente ofídico como uma doença tropical negligenciada prioritária e estima-se que 2,7 milhões de pessoas desenvolvam doença clínica após picada de cobra, com morbidade e mortalidade afetando, principalmente, indivíduos com menos de 30 anos de idade [17].

O Brasil tem o maior número de casos de picada de cobra na América do Sul – cerca de 26.000 a 29.000 por ano – seguido pela Venezuela (7.000), Colômbia (3.000), Equador (1.400-1.600), Peru (1.400-1.500) e Bolívia (1.000) [18]. Até junho de 2018, o Ministério da Saúde registrou 28.601 novos casos de ofidismo no Brasil [19].

Os acidentes envolvendo serpentes do gênero *Bothrops* são responsáveis por 90% dos envenenamentos, sendo de grande importância epidemiológica no Brasil. Esses acidentes são caracterizados por dor moderada ou severa e instalação de processo inflamatório e formação de edema intenso.

Observam-se também bolhas acompanhadas ou não de necrose. Como reações sistêmicas, têm-se equimoses e sangramentos [20].

É importante ressaltar que o tratamento convencional (soroterapia em combinação com fluido terapia, hemodiálise e antibióticos) não é capaz de reverter a dor dos acidentes ofídicos, portanto, não é suficiente para melhora da qualidade da assistência na sua totalidade [21]. Além disso, a morbidade crônica após acidentes ofídicos tem sido subdiagnosticada, muitas vítimas apresentam sintomas crônicos na região picada, incluindo síndrome dolorosa regional complexa (CRPA) [22, 23] e disfunções musculoesqueléticas [24]. Assim, tornam-se imprescindíveis pesquisas que levem ao desenvolvimento de terapias mais eficazes e capazes de tratar a dor inflamatória e crônica após o acidente ofídico.

Desta maneira, compreender e elucidar os mecanismos envolvidos na dor dos envenenamentos são necessários, pois a compreensão dos fatores moduladores neste fenômeno é essencial para o desenvolvimento de novas estratégias terapêuticas eficazes que possam colaborar com a melhor qualidade de tratamento dos pacientes.

1.2 Escorpionismo

O envenenamento por escorpiões é um problema de saúde pública emergente e negligenciado no mundo [12], mais de 1.2 milhões de envenenamentos por escorpiões ocorrem por ano no mundo [25]. No Brasil, de acordo com dados do Ministério da Saúde, em 2017, ocorreram 125 mil casos de picada de escorpiões no país [26].

No Brasil, existem cerca de 160 espécies de escorpiões, mas os acidentes com maior importância médica são causados pelo gênero Tityus [27]. Destes, duas espécies, *Tityus bahiensis* e *Tityus serrulatus*, são responsáveis pela maioria dos acidentes notificados de escorpião [12]. Esses acidentes são caracterizados por dor modera/severa, eritema, parestesia, sudorese, vômitos, taquipneia, aumento da pressão arterial, taquicardia ou bradicardia, podendo também manifestar sintomas compatíveis com insuficiência cardíaca congestiva aguda devido ao aumento da resistência vascular e edema agudo de pulmão [28].

O tratamento sintomático dos acidentes escorpiônicos baseia-se na administração do soro antiescorpiônico e/ou soro antiaracnídeo. Para o no alívio da dor a administração de lidocaína 2% sem vasoconstritor no local da picada ou usando dipirona ou outros analgésicos por via oral [12, 28]. Em um pequeno número de casos, a dor, intensa e persistente, permanece, frequentemente, como único sintoma [25].

No entanto, os mecanismos envolvidos no processo de dor induzido pelo envenenamento de escorpiões são pouco conhecidos. Dessa forma, a compreensão dos fatores moduladores neste fenômeno é essencial para o desenvolvimento de novas estratégias terapêuticas que sejam mais eficazes e propiciem uma assistência de melhor qualidade às vítimas de acidentes escorpiônicos.

1.3 Fisiopatogênese da dor

A capacidade de detectar estímulos potencialmente nocivos é conferida ao sistema somatosensorial e envolve interações complexas de mecanismos periféricos e centrais. A nocicepção refere-se à detecção e ao processamento de estímulos que podem vir a comprometer a integridade física do organismo como um todo. Dessa forma, o calor, o frio, a pressão, a distensão, os traumas, os estímulos químicos, dentre outros, podem, direta ou indiretamente, ativar os nociceptores e causar dor [29, 30].

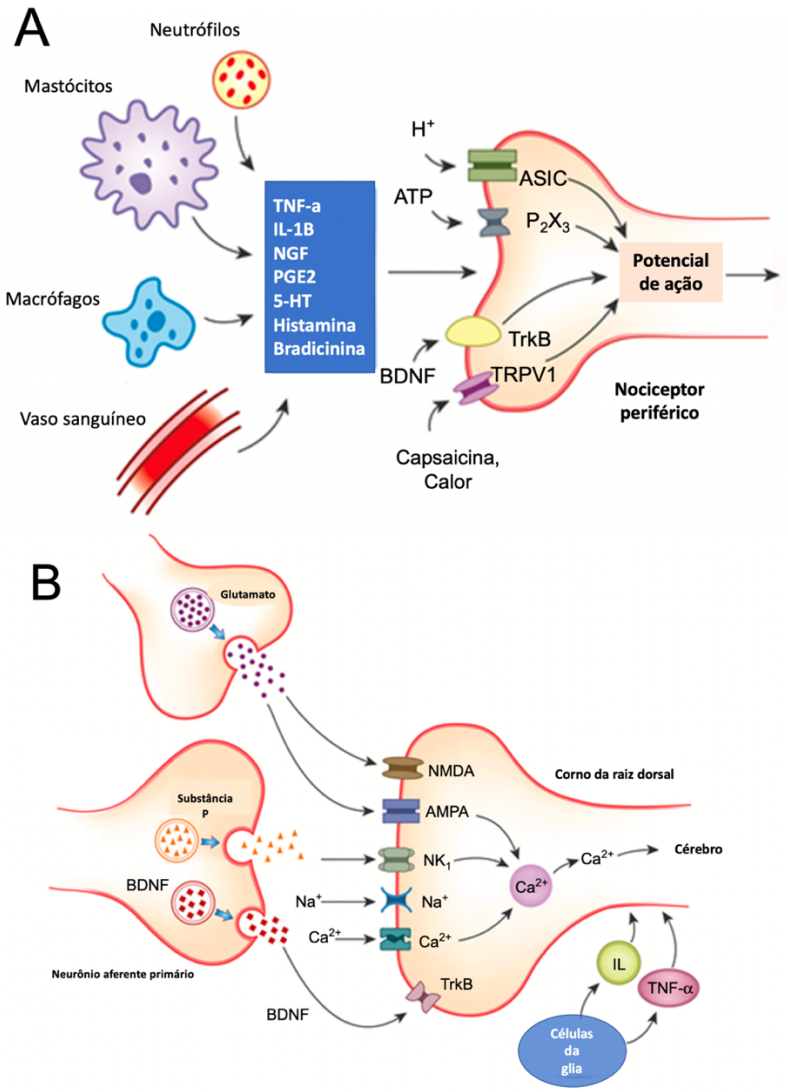
É necessário ressaltar que a dor nociceptiva é um tipo de dor de alto limiar, ou seja, requer um estímulo de alta intensidade para induzir a dor. Este tipo de dor possui caráter protetor, com um componente de aprendizagem que ajuda a evitar possíveis danos teciduais. Já a dor patológica é, principalmente, caracterizada pela mudança de um tipo de dor de limiar elevado para uma dor de baixo limiar, isto é, os estímulos não nocivos podem agora induzir dor.

A detecção do sinal nociceptivo é realizada por terminações periféricas livres de fibras aferentes primárias denominadas nociceptores e estas fibras sensoriais são responsáveis por propagar o sinal nociceptivo periférico para os neurônios secundários da medula espinhal. O neurônio nociceptivo primário transmite a informação periférica até o corno dorsal da medula espinal. A informação é transmitida ao neurônio nociceptivo secundário por meio da

liberação de neurotransmissores e mediadores pelo neurônio nociceptivo primário na fenda sináptica formada pelos dois neurônios. O neurônio nociceptivo secundário, por sua vez, conduz a informação direta ou indiretamente até os centros superiores localizados no encéfalo, onde são analisados e interpretados como dor [31] **(Figura 1).**

Assim, a ativação neuronal periférica é conduzida a medula espinal, a liberação de mediadores nesse local pode ativar as células da glia e desencadear uma resposta através da ativação das vias de sinalização e bem como a produção de mais mediadores inflamatórios, levando à manutenção da dor.

Figura 1: Mecanismo de sensibilização periférica e central.



(A) Ativação de nociceptores periféricos em resposta a estímulos, como calor, lesão ou distúrbios mecânicos, inicia-se a liberação de mediadores químicos no local da lesão (sensibilização

periférica). **(B)** A dor persistente ou inflamação causam ativação e disparo repetitivo em nociceptores aferentes de fibra C, que desencadeiam a liberação de glutamato no corno dorsal (sensibilização central). Isso é acompanhado pela liberação da substância P, BDNF e neurocininas, que causam despolarização persistente da membrana celular. Adicionalmente, levam a ativação de receptores AMPA ou NMDA por glutamato que estimula as células da glia e subsequentemente induz a liberação de COX 1 e 2, óxido nítrico e outros mediadores pró-inflamatórios (TNF-α, IL-1β, IL-6). Adaptado de Dureja *et al.,* 2017 [32].

1.3.1 Mecanismos periféricos celulares e moleculares na hiperalgesia: Recrutamento celular, citocinas pró-inflamatórias e fator de transcrição NFkB

A lesão tecidual/estímulo inflamatório é reconhecida por células residentes que liberam diferentes citocinas e quimiocinas. Estas moléculas desempenham papel chave no desencadeamento da dor inflamatória [33].

Em resposta a fatores quimiotáticos, neutrófilos migram da corrente sanguínea para o sítio inflamatório. No local da lesão, os neutrófilos liberam uma variedade de mediadores, incluindo mediadores lipídicos, quimiocinas e citocinas [34]. Os neutrófilos também atuam na indução da hiperalgesia ativando receptores específicos presentes nos nociceptores periféricos através da liberação de fatores como leucotrieno (LT) B4 e prostaglandina (PG) E2 [33, 35].

As citocinas, quimiocinas e outras moléculas, são 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 modelo experimental [36]. O fator de necrose tumoral-alfa (TNF-α) desempenha papel chave no desencadeamento da dor inflamatória [37]. Diferentes estímulos inflamatórios 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 indução 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 [36-38].

Dessa forma, é evidente a natureza multifatorial da dor de origem inflamatória, mediada por diferentes citocinas, que atuam paralelamente, em sequência ou até mesmo em sinergismo [39]. Em tempo, 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 [36].

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 [40]. Esta molécula 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 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, COX-2 e óxido nítrico sintase (iNOS) [41]. Além disso, foi demonstrado que o TNF-α e IL-1β também podem induzir a fosforilação e ativação do NFκB independente da degradação do IκB [42].

1.3.2 Mecanismos espinais celulares e moleculares na hiperalgesia: Células da glia e proteínas quinases ativadas por mitógenos (MAPK) na dor

Como dito acima, para que a sensação dolorosa ocorra é necessário a sensibilização dos nociceptores por mediadores durante o processo nociceptivo, estes mediadores são liberados por vários tipos celulares, como as células do sistema imune, neurônios e as células da glia. Na última década vários estudos demonstraram o envolvimento das células da glia na inflamação periférica e na dor.

As células da glia consistem, principalmente, em 3 grupos celulares: astrócitos, micróglia e oligodendrócitos e essas células são capazes de sintetizar várias substâncias que podem modular a dor, como por exemplo, prostaglandinas, glutamato, óxido nítrico e citocinas [43].

O primeiro relato sobre o papel das células da glia no processo nociceptivo foi evidenciado por Garrison e colaboradores em 1991, demonstraram o aumento da densidade das células da glia após ligadura do nervo ciático em

murinos [44]. Estudos posteriores demonstraram que os astrócitos e a micróglia, além de participarem dos processos inflamatórios, tem um papel importante no desenvolvimento e manutenção da dor [45-49].

De fato, vários estudos evidenciaram a participação dos astrócitos e da microglia na dor inflamatória. A inflamação periférica causa a sensibilização de neurônios espinais [50, 51] e reatividade de células gliais no corno dorsal da medula espinal [52]. Astrogliose (aumento da ativação de astrócitos) e microgliose (aumento da ativação de micróglia) espinal foram relatados em muitos modelos de dor inflamatória [53-56]. Em 2004, Chacur e colaboradores demonstram pela primeira vez o envolvimento das células da glia na dor induzida pela injeção periférica de fosfolipases do veneno de *Bothrops asper* em ratos [57, 58]. A inibição da atividade das células glia durante a inflamação periférica demonstrou ser significantemente importante para a redução da manifestação de dor em modelos experimentais [54, 56, 59], sugerindo que a atividade dessas células na medula espinal é vital para o desenvolvimento da dor inflamatória.

Na medula espinal, estímulos periféricos causam a liberação de citocinas pró-inflamatórias, como TNF-α e IL-1β pelas células da glia [43]. TNF-α e IL-1β são regulados positivamente na medula espinal em modelo de lesão de nervo periférico, inflamação e câncer ósseo. Portanto, estas citocinas contribuem para o desenvolvimento e manutenção da dor inflamatória, neuropática e câncer [46, 60-62].

O TNF-α é produzido, principalmente, pela micróglia e desempenha um papel essencial na geração da sensibilização central e dor persistente [63-66], além do seu papel bem documentado na modulação da sensibilização periférica [67-69]. A IL-1β é produzida em astrócitos após câncer ósseo, inflamação e lesão do nervo [70-73].

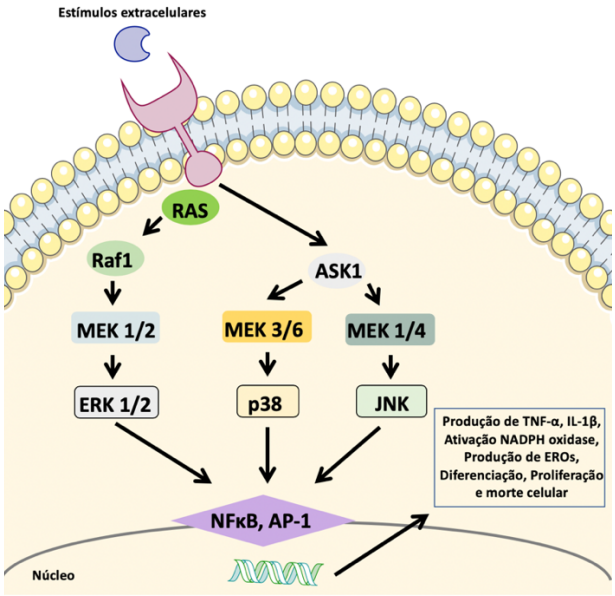
A IL-1β também pode ser produzida por micróglia e neurônios na medula espinhal [63, 74, 75]. A inibição da sinalização da IL-1β espinhal tem sido eficaz na redução da dor inflamatória, neuropática e oncológica [70, 71, 73, 76, 77], sendo que a sinalização intracelular para produção de TNF-α e IL-1β na medula espinal é regulada pela ativação do fator de transcrição nuclear NFkB tanto nos astrócitos quanto na micróglia [78-80].

As proteínas quinases ativadas por mitógeno (MAPKs) tem um papel crítico na cascata de transdução de sinal associada à nocicepção. A família de

MAPK inclui 3 membros principais: quinase regulada por sinal extracelular (ERK), p38 e c-Jun quinases N-terminais (JNK) (**Figura 2**). A via da MAPK desempenha um papel importante na sinalização intracelular nas células glia para o desenvolvimento e manutenção da dor [81, 82]. Curiosamente, diferentes MAPKs exibem padrões distintos de ativação (fosforilação) nas células da glia [81].

Assim, temos os mecanismos astrocíticos e microgliais de ativação de MAPKs. Os mecanismos astrocíticos incluem a fosforilação de JNK e ERK espinal por estímulos periféricos [72, 83]. A injeção intraplantar de CFA [72, 83] ou carragenina [84] induz a fosforilação da ERK e JNK e a inibição da JNK atenua os comportamentos de dor [83, 84]. A fosforilação da JNK é regulada pela secreção espinal de TNF-α [84] como é o caso durante a dor neuropática [83]. Já fosforilação da ERK é regulada pela secreção espinal de IL-1β [72]. O mecanismo microglial espinhal que regula a dor inflamatória é a fosforilação da p38. A inflamação periférica induz extensa fosforilação da p38 que é restrita às células da micróglia [85, 86]. A inibição da p38 em modelos de dor inflamatória resulta na atenuação dos comportamentos de dor [85, 86], dessa forma, a ativação de vários receptores microgliais resulta em fosforilação da p38 intracelular, sugerindo que esta é uma via de sinalização intracelular chave durante a dor inflamatória e neuropática [87, 88].

Figura 2: Via de sinalização das MAPK (ERK, JNK e p38).



Fonte: Próprio autor.

1.4 Jararagina, uma metaloprotease do veneno de *Bothrops jararaca*

No reino animal, os venenos são usados principalmente como parte da estratégia predatória para paralisar, capturar e matar presas ou como um mecanismo de defesa para impedir predadores e/ou competidores [89].

O envenenamento por *Bothrops jararaca* é seguido de dor severa e inflamação, e ainda se associam a estes sintomas: hemorragia, edema e mionecrose, que se instalam no local da picada, resultando em sérias complicações clínicas, tais como sequelas permanentes ou até mesmo a amputação do membro afetado [20]. O tratamento das vítimas é realizado pela administração da soroterapia, que neutraliza com eficiência os efeitos sistêmicos do acidente ofídico, tais como alterações na coagulação sanguínea e cardiovasculares, choque hipovolêmico e alterações renais. No entanto, os efeitos locais como hemorragia, necrose, dor e edema não são neutralizados com eficiência [21].

Dada a importância epidemiológica dos acidentes ofídicos por *Bothrops jararaca* e as limitações na eficácia das formas de tratamento convencionais, é importante o desenvolvimento de novas abordagens terapêuticas para as vítimas de acidente botrópico.

O primeiro relato sobre o efeito hiperalgésico de venenos botrópicos foi descrito em 1994 [15], sendo que a hiperalgesia induzida pelo veneno de *Bothrops jararaca*, serpente amplamente distribuída nas regiões Sul e Sudeste do Brasil, é mediada por prostangladinas, leucotrienos e fator de agregação plaquetária (PAF). Posteriormente, com a participação da bradicinina [90] e aminas biogênicas [91] também foram demonstrados altos níveis de citocinas pró-inflamatórias no sangue de pacientes envenenados por essa serpente [92].

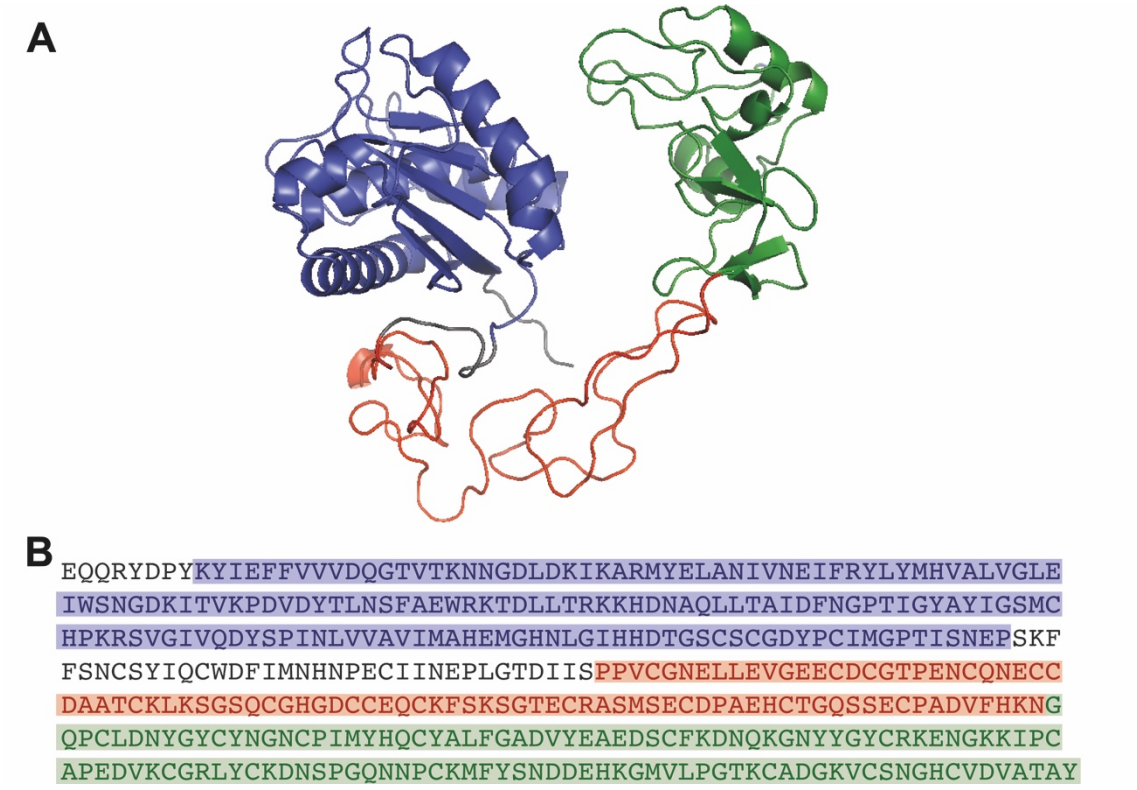
É necessário lembrar que venenos são coquetéis complexos de sais, nucleotídeos, aminoácidos livres, neurotransmissores, poliaminas, peptídeos e proteínas. Do ponto de vista farmacológico, as proteínas tem atraído o maior interesse e há um significativo número de estudos que visam investigar os mecanismos de ação em receptores-alvo [93].

Ao analisar os componentes isolados do veneno de *Bothrops jararaca*, verificou-se que as metaloproteinases de veneno de serpentes (SVMPs) correspondem a 51,5% da composição do veneno [94, 95], e esta classe de

proteína está envolvida na resposta local do envenenamento, além de ser a principal responsável pela hiperalgesia induzida pelo veneno de *Bothrops jararaca* [96]. Dentre as SVMPs do veneno de *Bothrops jararaca*, a mais bem caracterizada, bioquímica e biologicamente, é a jararagina [97].

A jararagina é uma metaloproteinase hemorrágica com massa de 52 kDa. (421 aminoácidos) que contém três domínios: um domínio catalítico, um domínio semelhante a disintegrina e um domínio rico em cisteína (**Figura 3**).

Figura 3: Estrutura tridimensional e sequência linear da jararagina.



(A) Estrutura tridimensional da jararagina (UniProt: P30431) obtida por modelagem por homologia usando a proteína *Bothropasin* (PDB 3DSL) como template. (**B)** Sequência linear de aminoácidos da jararagina (UniProt: P30431). Domínio catalítico (azul), semelhante a disintegrina (vermelho) e rico em cisteína (verde). Fonte: Próprio autor.

A jararagina é responsável por muitos efeitos do veneno de *Bothrops jararaca.* Por exemplo, a jararagina contribui para o efeito anticoagulante pela clivagem do fibrinogênio [98], degradando a fibrina [99], inibindo a agregação plaquetária induzida por colágeno e ristocetina [100] e inibindo a agregação

plaquetária induzida por colágeno [101]. De fato, a jararagina é capaz de induzir a produção de citocinas, como TNF-α e IL-1β *in vivo* [102, 103] e expressão de mRNA de TNF-α e IL-1β *in vitro* [104]. Também foi demonstrado que a dor induzida pela a jararagina é mediada pela produção de TNF-α e IL-1β e NFkB *in vivo* [105].

Nesse sentido, a investigação de novos alvos farmacológicos envolvidos no processo de dor induzido pela jararagina pode ser a chave para o desenvolvimento de novas drogas e terapias para o tratamento da dor nos acidentes ofídicos, bem como compreender os mecanismos ativados durante o envenenamento.

Até o momento, não há trabalhos que investigaram os possíveis [106]mecanismos espinais ativados pela jararagina, todavia há indícios que corroboram nossa hipótese que componentes de veneno de serpentes são capazes de ativar mecanismos e células espinais, como demonstrado por Chacur e colaboradores [58, 90].

1.5 Potencial hiperalgésico dos venenos de *Tityus bahiensis* e *Tityus serrulatus*

Os venenos de animais evoluíram ao longo de milhões de anos para capturar as presas e/ou defesa de predadores e rivais [107]. O veneno de escorpião é uma mistura de peptídeos, incluindo potenciadores de bradicinina e peptídeos e proteínas pequenas a médias, como metaloproteinases e fosfolipases que sinérgica ou isoladamente causam as manifestações clínicas, incluindo a dor [108, 109].

Os estudos com os venenos de *Tityus bahiensis* e *Tityus serrulatus* buscam compreender os efeitos sistêmicos induzidos pelo o envenenamento, como neurotoxicidade [106] e insuficiência respiratória [110] e na busca de potenciais toxinas analgésicas.

Dessa forma, os mecanismos inflamatórios e nociceptivos dos venenos de *Tityus bahiensis* e *Tityus serrulatus* ou de suas moléculas isoladas são pouco estudados.

O veneno de *Tityus bahiensis* induz edema e recrutamento de leucócitos em ratos [111], e o Tb II-I, uma proteína isolada do veneno de *Tityus bahiensis*, foi capaz de aumentar os níveis de IL-6 e TNF-α em ratos [112].

Por sua vez, o veneno de *Tityus serrulatus* causa edema, hiperalgesia mecânica de forma dose-dependentes, devido ao aumento de eicosanóides, histamina e 5-hidroxitriptamina [111, 113]. O Ts8, um peptídeo isolado do veneno de *Tityus serrulatus*, modula os canais de Kv4.2 induzindo dor espontânea e hiperalgesia mecânica [31]. E Ts2 e Ts6, proteínas isoladas do veneno de *Tityus serrulatus*, induzem migração celular de neutrófilos, leucócitos e células mononucleares e produção de citocinas pró-inflamatórias [114].

Dessa forma, a compreensão dos fatores moduladores na dor induzida pelos venenos de escorpiões é essencial para o desenvolvimento de novas estratégias terapêuticas mais eficazes para esses acidentes.

2 OBJETIVOS

2.1 Objetivo Geral

Elucidar os mecanismos periféricos e espinais (celulares e moleculares) envolvidos na nocicepção induzida pela metaloproteinase *do veneno de Bothrops jararaca*, jararagina, e pelo veneno de *Tityus serrulatus* e *Tityus bahiensis.*

2.2 Objetivos Específicos

- Avaliar o perfil temporal da produção de TNF-α e IL-1β espinal após administração periférica de jararagina;
- Avaliar a participação do TNF-α, IL-1β e NFκB espinais na hiperalgesia induzida pela jararagina;
- Avaliar a participação do NFκB na produção de TNF-α e IL-1β induzidos pela jararagina;
- Avaliar o envolvimento das células da glia e das MAPKs na hiperalgesia induzida pela jararagina;
- Avaliar o perfil temporal de ativação das células da glia e das MAPKs espinais após injeção periférica de jararagina;
- Avaliar o perfil temporal da hiperalgesia mecânica e térmica após administração do veneno de *Tityus serrulatus* e *Tityus bahiensis;*
- Avaliar o perfil temporal do recrutamento celular periférico após administração do veneno de *Tityus serrulatus* e *Tityus bahiensis;*
- Avaliar a participação do TNF-α, IL-1β e NFκB periféricos na nocicepção induzida pelo veneno de *Tityus serrulatus* e *Tityus bahiensis.*

3 MATERIAL E MÉTODOS

3.1 Animais

Os experimentos foram realizados utilizando camundongos machos da linhagem Swiss (20 a 25g). Os camundongos foram alocados em caixas na rack ventilada para camundongos (IVC, AL22, Alesco®). Os camundongos foram mantidos no biotério em ciclo claro/escuro (12/12h), com livre acesso a água e ração. Os experimentos comportamentais foram realizados entre 9:00 e 17:00 em uma sala com temperatura controlada (24±2 ºC). Foram realizadas duas repetições para cada modelo experimental. Foi utilizado o n=6 de animais por grupo. Todos os experimentos foram conduzidos de acordo com Associação Internacional do Estudo da Dor (IASP) e com as normas estabelecidas pelo Comitê de Ética em Experimental Animal da Universidade Estadual de Londrina, Nº 7786.2014.42, 2519.2015.83 e 21366.2015.72.

3.2 Purificação da jararagina

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. 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) [115] e modificada por Moura-da-Silva e colaboradores (2003) [116].

3.3 Venenos de *Tityus bahiensis* e *Tityus serrulatus*

Os venenos liofilizados de *Tityus bahiensis* e *Tityus serrulatus* foram fornecidos pelo Instituto Butantan (São Paulo, Brasil), e foram mantidos a -20 °C até o momento do uso.

3.4 Protocolos experimentais e tratamentos

3.4.1 Jararagina

A jararagina (1 µg/pata) foi administrada via intraplantar (i.pl.). Animais controles receberam a injeção i.pl de solução salina. Para avaliação da produção de citocinas (TNF-α e IL-1β), os camundongos foram eutanasiados com isofluorano inalatório e a coleta do tecido espinal (L4-L6) de cada grupo experimental foi realizada nos tempos 1, 3, 5 e 7 horas após o estímulo. A avaliação do efeito do PDTC na produção de citocinas foi realizado na 3° hora. Para o ensaio de Western blot as amostras do tecido espinal (L4-L6) foram coletadas de cada grupo experimental nos tempos 1, 3, 5 e 7 horas (MAPKs) e 1, 3, 5, 7 horas (células da glia) após estímulo. Para o ensaio de RT-qPCR coleta do tecido espinal (L4-L6) de cada grupo experimental foi realizada nos tempos 1, 3, 5, 7 horas (células da glia) após o estímulo.

Para a avaliação dos diferentes inibidores foi realizado o pré- tratamento intratecal (i.t.) com: etanercept (Enbrel®) da Wyeth Indústria Farmacêutica Ltda (São Paulo, Brazil), 100 µg/5 µL i.t., 30 minutos antes da administração da jararagina; IL- 1ra do NIBSC (National Institute of Biological Standards and Control, UK), 100 pg/5 µL i.t., 30 minutos antes do estímulo, PDTC da Santa Cruz Biotechnology (Dallas, Estados Unidos da América), 300 µg/5 µL i.t., 30 minutos antes do estímulo, minociclina da Sigma-Aldrich (St. Louis, MO, USA), 50 µg/5 µL i.t., 30 minutos antes do estímulo, α-aminoadipate da Sigma-Aldrich (St. Louis, MO, USA), 100 nmol/5 µL i.t., 30 minutos antes do estímulo, PD 98059 da Sigma-Aldrich (St. Louis, MO, USA), 1, 3 ou 10 µg/5 µl, i.t., 30 minutos antes do estímulo, SB 202190 da Sigma-Aldrich (St. Louis, MO, USA), 1, 3 ou 10 µg/5 µl, i.t., 30 minutos antes do estímulo, e SP 600125 da Sigma-Aldrich (St. Louis, MO, USA), 1, 3 ou 10 µg/5 µl, i.t., 30 minutos antes do estímulo. As doses foram padronizadas previamente no nosso laboratório em experimentos preliminares. Jararagina, etanercept e IL1-ra foram dissolvidos em salina, minociclina e PDTC foram dissolvidos em 2% de DMSO em salina, α-aminoadipate foi dissolvido em 20% de tween mais 2% de DMSO em salina e SP 600125, PD 98059 e SB 202190 foram dissolvidos em 20% de DMSO em salina. Após o estímulo com a jararagina (1µg/pata) os animais foram avaliados quanto à hiperalgesia mecânica nos tempos 1, 3, 5 e 7 horas.

3.4.2 Venenos de Tityus bahiensis e Tityus serrulatus

Os venenos *de Tityus bahiensis* e *Tityus serrulatus* foram administrados via subcutânea i.pl. nas doses de 0.2, 0.6, 1.2 e 2.4 µg/20 µL, sendo escolhida a dose de 2.4 µg para os experimentos posteriores. Animais controles receberam a injeção i.pl de 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 0.5, 1 e 3 horas após o estímulo e para avaliação indireta do recrutamento de neutrófilos (MPO) e macrófagos (NAG) o tecido plantar foi coletado 5 horas após estímulo. A dor espontânea foi avaliada por 30 min após administração i.pl. dos venenos.

Para a avaliação com os diferentes inibidores foi realizado o pré-tratamento com etanercept (Enbrel®) da Wyeth Indústria Farmacêutica Ltda (São Paulo, Brazil), 10 mg/Kg, 200 µL, i.p., pré-tratamento 48 horas e 1 hora antes do estímulo, IL- 1ra do NIBSC (National Institute of Biological Standards and Control, UK), 30 mg/Kg, 200 µL, i.p. pré-tratamento 30 minutos antes do estímulo e PDTC da Santa Cruz Biotechnology (Dallas, Estados Unidos da América), 100 mg/Kg, 100 µL s.c. pré-tratamento 30 minutos antes do estímulo (doses padronizadas no laboratório em experimentos preliminares) [105]. Após o estímulo com os venenos os animais foram avaliados quanto à hiperalgesia mecânica e térmica nos tempos 0.5, 1, 3, 5 horas, dor espontânea e atividade da MPO e NAG.

3.5 Hiperalgesia mecânica

A avaliação da hiperalgesia mecânica foi realizada pelo método de von Frey, modificado por Cunha et al. (2004) [117], 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 dos estímulos e nos intervalos de tempo determinados. Para cada tempo, foi considerada a média de três medições. Os resultados foram relatas 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.

3.6 Hiperalgesia térmica

Para este experimento, os camundongos serão transferidos para placa de metal mantida por uma fonte de calor a 55°C. Inicialmente, serão realizadas duas medidas controle em intervalos de 30 minutos, para estabelecer o tempo máximo de permanência do animal sobre a placa. A resposta ao estimulo térmico será avaliada pela cronometragem do número de retiradas e lambidas das patas. Em seguida, será realizada a inoculação intraplantar do estímulo e as medidas do tempo de resposta ao estímulo térmico serão registradas em intervalos de 30 minutos. Os resultados serão expressos em média e desvio padrão dos tempos registrados nos diferentes grupos [118].

3.7 Dor espontânea (sacudida de pata e tempo gasto ao lamber a pata)

O número de sacudidas da pata e o tempo gasto ao lamber a pata foram determinados entre 0-30 min após injeção i.pl. do estímulo [118].

3.8 Avaliação da atividade da mieloperoxidase (MPO)

A quantificação da migração de neutrófilos ao tecido subcutâneo plantar foi realizada indiretamente pela atividade da mieloperoxidase (MPO). Após administração do estímulo, o tecido subcutâneo plantar foi coletado em tampão fosfato de potássio 50 mM contendo HTAB (Brometo de hexadecil trimetil amônio) 13,72 mM, pH 6.0, na concentração de 50 mg de tecido/mL de tampão. As amostras foram homogeneizadas com auxílio do Polytron (PT3100), seguida de centrifugação (2 min, 16.100 g, a 4º C). A dosagem por reação colorimétrica cinética foi realizada em placas de 96 poços contendo os sobrenadantes das amostras e 200 µL da solução de reação (52,64 mM de dihidrocloreto de O-

dianisidina, 0,05% de H2O2 30%, 90 mL de tampão fosfato de potássio 50 mM, pH 6.0 e 10 mL de H2O destilada). A leitura foi realizada a 450 nm (Spectra MAX 250, Molecular Devices). Os resultados foram expressos como número de neutrófilos por miligrama de tecido, utilizando-se uma curva padrão de neutrófilos [119, 120].

3.9 Avaliação da atividade da enzima N-acetil-β-D-glicosaminidase (NAG)

Para a atividade de NAG, 20 µL de sobrenadante, anteriormente descrito na atividade de MPO, foram colocados numa placa de 96 poços e em seguida adicionar 80 µL de tampão sem HTAB. A reação foi iniciada pela adição de 25 µL de 4-Nitrofenil-N-acetil-β-D-glicosaminidase 2,24 mM. A placa foi incubada a 37 °C durante 1 h e a reação parada pela adição de 30 µL de 200 nM de glicina, pH 10,4. A atividade enzimática foi determinada colorimetricamente usando um leitor de placa a 405 nm. Os resultados foram expressos como número de macrófagos por miligrama de tecido, utilizando-se uma curva padrão de macrófagos [119].

3.10 Dosagens de TNF-α e IL-1β

Amostras de tecido espinhal (L4-L6) ou tecido plantar foram coletadas nos momentos indicados após estímulo e homogeneizadas com ultraturrax (ULTRA-TURRAX®) em tampão contendo inibidores de protease para dosagem dos níveis de TNF-α e IL-1β usando kits ELISA comerciais de acordo com as instruções do fabricante (eBioscience®, Ready-SET-Go).

3.11 RT-PCR e PCR quantitativo

Amostras de tecido espinal (L4-L6) foram homogeneizadas em reagente TRIzol® (Life Technologies) e o RNA total foi isolado de acordo com as instruções do fabricante. A pureza do RNA foi confirmada pela razão 260/280. A RT-PCR e PCR quantitativo foram realizadas usando o sistema RT-qPCR de 2 passos GoTaq® (Promega) em um sistema de PCR em tempo real StepOnePlus ™ (Applied Biosystems®).

Sequências dos primers:

Iba-1	5’- ATGGAGTTTGATCTGAATGGAAAT-3’
	5’- TCAGGGCAGCTCGGAGATAGCTTT-3’
Gfap	5’- GGCGCTCAATGCTGGCTTCA-3’
	5’- TCTGCCTCCAGCCTCAGGTT-3
Gapdh	5’- CATACCAGGAAATGAGCTTG-3’
	5’- ATGACATCAAGAAGGTGGTG-3’

3.12 Western blot

Amostras de tecido espinhal (L4-L6) foram coletadas nos momentos indicados após estímulo com jararagina e homogeneizados em tampão RIPA contendo inibidores de protease e fosfatase. Os lisados foram então homogeneizados e centrifugados (0,5 g durante 10 min a 4 ° C). Os extratos de proteína foram separados por SDS-PAGE e transferidos para a membrana de nitrocelulose para a outra análise (GE Healthcare-Amersham, Pittsburgh, PA, EUA). As membranas foram então incubadas em tampão de bloqueio [albumina de soro bovino a 5% (BSA) ou leite a 5%] durante tempos diferentes para cada anticorpo a 4ºC e incubadas overnigt a 4ºC na presença do anticorpo. Anticorpos utilizados: anti-fosfo p38 (sc17852, 1:100) e total p38 (sc535, 1:100); anti-fosfo-JNK (#4671, 1:500) e total JNK (#9252, 1:300) e anti- β-actina (1: 10.000; Sigma-Aldrich) foi usado para controle. As massas moleculares das proteínas foram confirmadas pelo padrão de proteína (Bio-Rad, Hercules, CA, EUA). Após lavagem em TBS com Tween 20, a membrana foi incubada com anticorpo secundário durante 2 h à temperatura ambiente. A proteína foi visualizada por quimiluminescência com reagente de detecção ECL (GE Healthcare-Amersham).

3.13 Análise Estatística

Os resultados são apresentados como média ± SEM das medições feitas em 6 camundongos em cada grupo por experimento, representativos de 2

experimentos independentes. A análise de variância bidirecional (Two-way ANOVA) seguida pelo teste de Tukey foi usada quando as respostas foram medidas em diferentes momentos após a injeção do estímulo. A analise de variância (One-way ANOVA) seguido pelo teste de Tukey foi realizado para comparar os valores nos pontos de tempo indicados. Em todos os cálculos foi fixado o nível de significância de 5% ($p<0,05$) usando o software Prism 6.0.

4 RESULTADOS E DISCUSSÃO

Os resultados obtidos no doutoramento em Ciências da Saúde estão descritos em três artigos científicos que serão submetidos à revista Toxins, com Qualis-periódicos A2 em medicina I e fator de impacto 3.2, com os títulos:

4.1 Spinal cord microglia and astrocyte mediate jararhagin-induced mechanical hyperalgesia in mice.

Camila Rodrigues Ferraz, Thacyana Teixeira de Carvalho, Felipe Almeida Pinho Ribeiro, Victor Fattori, Thiago Mattar Cunha, Fernando Queiroz Cunha, Rúbia Casagrande, Fábio Henrique Kwaskiewski, Patricia Bianca Clissa, Waldiceu Aparecido Verri

4.2 Jararhagin-induced mechanical hyperalgesia depends on spinal activation of MAP kinases in mice.

Camila Rodrigues Ferraz, Thacyana Teixeira de Carvalho, Thiago Mattar Cunha, Fernando Queiroz Cunha, Rúbia Casagrande, Patricia Bianca Clissa, Waldiceu Aparecido Verri

4.3 Peripheral mechanisms involved in the nociception triggered by *Tityus bahiensis* and *Tityus serrulatus* venom.

Camila Rodrigues Ferraz, Marília Fernandes Manchope, Ketlem Cristina Andrade, Rúbia Casagrande, Fábio Henrique Kwaskiewski, Waldiceu Aparecido Verri

Os artigos seguem as normas solicitadas pela revista.

4.1 Spinal cord microglia and astrocyte mediate jararhagin-induced mechanical hyperalgesia in mice

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ABSTRACT

Jararhagin is a hyperalgesic metalloproteinase from *Bothrops jararaca* venom. In rodents, jararhagin induces nociceptive behaviors that correlate with increase on peripheral cytokine levels. However, the role of the spinal cord in pain processing after peripheral injection of jararhagin has not been investigated. Mice received intraplantar (i.pl.) injection of jararhagin and the following parameters were evaluated: hyperalgesia, spinal cord TNF-α, IL-1β levels, NFκB activation, and Iba-1 and GFAP expression. The effects of intrathecal (i.t.) injection of TNF-α soluble receptor (etanercept), IL-1 receptor antagonist (IL-1ra), and inhibitors of NFκB (PDTC), microglia (minocycline) and astrocyte (α-aminoadipate) were investigated. Jararhagin injection induced cytokine production (TNF-α and IL-1β) in the spinal cord, which was reduced by treatment with PDTC (40% and 50%, respectively). Jararhagin mechanical hyperalgesia and cytokine production were inhibited by treatment with etanercept (60%), IL-1ra (45%), PDTC (65%), minocycline (48%) and α-aminoadipate (40%). Furthermore, jararhagin induced an increase of Iba-1 and GFAP. These results demonstrate that jararhagin-induced mechanical hyperalgesia is dependent on spinal cord activation of glial cells and consequent NFκB activation and cytokine production in mice.

Keywords

Jararhagin, Metalloproteinase, *Bothrops jararaca*, Hyperalgesia, Spinal glia, Cytokines, NFκB

1 Introduction

Venoms play a crucial role for venomous animal defense [1, 2]. Components present in the venoms activate cells, receptors and signaling pathways inducing pain and inflammation. Snake envenomation is a significant health and economic burden worldwide [3]. Snakebite is a devastating environmental and occupational disease, especially in rural areas of tropical countries such as Brazil [4-6], with reports indicating approximately 27.000 snakebites each year [7].

Bothrops jararaca is a medically-important viper snake and the main responsible for envenomation in Brazil due to its abundance and broad geographical distribution [8-10]. *Bothrops jararaca* bite causes severe local pain [11, 12] which is not reversed by the antivenom [13]. At same time, chronic morbidity following snakebites have been underdiagnosed, with many victims reporting chronic symptoms in the bitten region, including complex regional pain syndrome (CRPA) [14, 15] and musculoskeletal disabilities [16]. Metalloproteases are the major components of *Bothrops jararaca* venom [17, 18] and the main responsible for local tissue damage, haemorrhage, coagulopathy [19-21] and pain induced by the snakebite [22].

Jararhagin is a metalloproteinase isolated from *Bothrops jararaca* venom [23]. Several studies have showed the involvement of jararhagin in local damage [18, 24], inflammatory response [24-26] and pain [27, 28]. However, the mechanisms underlying jararhagin-induced pain are poorly understood. So far is known that this metalloprotease induces the production of pro-inflammatory cytokines (TNF-a and IL-1β) *in vivo* [17, 26, 29] and *in vitro* [25]. In addition, it

has been shown that mechanical hyperalgesia induced by jararhagin [27, 28] depends on peripheral production of the cytokines TNF-α and IL-1β and activation of the transcription factor NFκB [27], which regulates cytokine production [30-36]. This is particularly important because peripheral inflammation induces changes in the spinal cord circuit that may lead to central sensitisation [37, 38]. Despite efforts in identifying peripheral mechanisms related to jararhagin-induced pain, involvement of spinal cord glial cells has not yet been described.

Glial cells make up over 70% of the total cell population in the central nervous system (CNS), and are classified into astrocytes, oligodendrocytes, and microglia [39]. Glial cells interact with nociceptor sensory neurons regulating neuronal processing in the spinal cord and contributing to the facilitation of pain signalling [40, 41]. Several studies support a role for spinal cord microglia and astrocytes in pain mechanisms, and showed peripheral inflammation activates glial cells [42-45]. For instance, *Bothrops asper venom* is able to induce glial cells activation and peripheral injection of isolated phospholipases from *Bothrops asper* venom induces mechanical hyperalgesia as well as activation of microglia and astrocytes. Altogether, these data suggest that snake venom components facilitate pain responses with the contribution of mechanisms related to the activation of glial cells [46].

The present data show that jararhagin-induced peripheral hyperalgesia in mice activates microglia and astrocyte in the spinal cord. We also observed a glial cells/NFκB-dependent production of TNF-α and IL-1β in the spinal cord, and this mechanism explains, at least in part, the mechanical hyperalgesia triggerd

by peripheral jararhagin. Conceptually, these data also suggest the role of spinal cord mechanisms in the development and maintenance of pain in snakebites.

2. Methods

2.1 Animals

The experiments were performed on male swiss mice (20-25g, State University of Londrina, Londrina, PR, Brazil) housed in individually ventilated caging (IVC) rack for mice (AL22, Alesco®) 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 (24±2 ^{o}C). 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 Londrina State University (CEUA Nº 7786.2014.42 and 2519.2015.83).

2.2 Jararhagin purification

The jararhagin was purified as described [27] in the Laboratory of Immunopathology, Butantan Institute. Jararhagin was subjected to treatment with Triton X-114 as described by Aida and Pabst (1990) [47] to remove any possible contamination with LPS. Limuls Amebocyte Lysate (LAL) test was performed to confirm absence of LPS.

2.3 Drug administration and drugs

The treatments (etanercept, interleukin-1 receptor antagonist, pyrrolidine dithiocarbamate, minocycline, and α-aminoadipate) were performed by

intrathecal (i.t.) injections described [48] and jararhagin stimulus by intraplantar (i.pl.) injections [27].

Drugs used were: Jararhagin (1 µg/20 µL i.pl.) [27] minocycline (50 µg/5 µL i.t., 30 min before stimulus) [49], interleukin-1 receptor antagonist (IL-1ra) (100 pg/5 µL i.t., 30 min before stimulus) from NIBSC (National Institute of Biological Standards and Control, UK), etanercept (100 µg/5 µL i.t., 30 min before stimulus) from Wyeth Indústria Farmacêutica Ltda (São Paulo, Brazil), pyrrolidine dithiocarbamate (PDTC) (300 µg/5 µL i.t., 30 min before stimulus) from Santa Cruz Biotechnology, α-aminoadipate (100 nmol/5 µL i.t., 30 min before stimulus) from Sigma-Aldrich (St. Louis, MO, USA). Jararhagin, etanercept and IL1-ra were dissolved in saline, minocycline, and PDTC were dissolved in 2% DMSO in saline, α-aminoadipate was dissolved in 20% tween, 2% DMSO in saline.

2.5 Experimental protocols

Mechanical hyperalgesia was evaluated after 1, 3, 5, and 7 h after an intraplantar (i.pl.) injection of jararhagin (1 µg/paw) or saline. Regarding the effect of pharmacological treatments over mechanical hyperalgesia mice were treated with minocycline (50 µg/5 µL, i.t., 30 min before stimulus), IL-1ra (100 pg/5 µL i.t., 30 min before stimulus), etanercept (100 µg/5 µL i.t., 30 min before stimulus), PDTC (300 µg/5 µL i.t., 30 min before stimulus), α -aminoadipate (100 nmol/5 µL i.t., 30 min before stimulus) or saline. Cytokine (TNF-α and IL-1β) levels were determined 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 minocycline (50 µg/5 µL, i.t., 30 min before stimulus), PDTC (300 µg/5 µL i.t., 30 min before stimulus) and α-aminoadipate (100 nmol/5 µL i.t., 30 min before stimulus). Spinal glia cell

activation (Iba-1 and GFAP mRNA expression) determined 1, 3, 5, and 7 h after jararhagin injection (1 µg/paw) by RT-qPCR. Doses of treatment were based on previous studies of our group and standardization in our laboratory [48, 49].

2.6 Electronic pressure–meter test for mice

Mechanical hyperalgesia was tested in mice by the electronic von Frey anesthesiometer: Insight, Ribeirão Preto, SP, Brazil adapted with a 0.5 mm^2 contact area polypropylene tip as previously reported [50]. The results are expressed by delta (∆) withdrawal threshold (in grams), which was calculated by subtracting the zero-time mean measurements from the mean measurements after stimulus at the indicated time points.

2.7 Cytokine measurement

Spinal tissue samples of L4–L6 segments were collected at indicated time points after stimulus with jararhagin, and homogenized using a ultraturrax (ULTRA-TURRAX®) 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).

2.8 RT-PCR and quantitative PCR

Spinal tissue samples of L4–L6 segments were homogenized into TRIzol® reagent (Life Technologies), and total RNA was isolated according to manufacturer’s directions. RNA purity was confirmed by the 260/280 ratio. RT-PCR and quantitative PCR were performed using GoTaq® 2-Step RT-qPCR System (Promega) on a StepOnePlus™ Real-Time PCR System (Applied

Biosystems®). Primer sequences: Iba-1 forward 5'-ATGGAGTTTGATCTGAATGGAAAT-3', reverse 5'-TCAGGGCAGCTCGGAGATAGCTTT-3'; Gfap forward 5'-GGCGCTCAATGCTGGCTTCA-3', reverse 5'-TCTGCCTCCAGCCTCAGGTT-3'; Gapdh forward 5'-CATACCAGGAAATGAGCTTG-3', reverse 5'-ATGACATCAAGAAGGTGGTG-3'.

2.9 Statistical analysis

Results are presented as means ± SEM of measurements made on 6 mice in each group per experiment, representative of 2 independent experiments. Two-way repeated measure analysis of variance (ANOVA) followed by Tukey's post hoc was used when responses were measured at different times points after the stimulus injection. One-way ANOVA followed by Tukey's post hoc test was performed to compare the values at the indicated time points. Statistical differences were significant when $P < 0.05$ in the software Prism 6.0.

3. Results

3.1 Peripheral jararhagin injection increased TNF-α and IL-1β levels in spinal cord

Peripheral stimulus induces cytokine production in the spinal cord. Based on that, we first investigated whether peripheral injection of jararhagin induces cytokine production. Jararhagin induced significantly TNF-α production at 3 h after its injection (Fig. 1A) and IL-1β production at 1, 3, and 5 h (Fig. 1B). These

results showed that peripheral injection of jararhagin was able to induced production of spinal cytokines.

3.2 Intrathecally targeting TNF-α, IL-1β, and NFκB *reduce jararhagin induced mechanical hyperalgesia*

Given we observed an increase on TNF-α and IL-1β we next investigated whether jararahagin-induced pain depends on these cytokines. These cytokines are produced in an NFκB-dependent manner, therefore, we also tested if inhibiting NFκB activation reduces pain in this model. Etanercept (Fig. 2A), IL-1ra (Fig. 2B) and PDTC (Fig. 2C) inhibited jararhagin-induced mechanical hyperalgesia at all time points evaluated indicating the role of TNF-α, IL-1β and NFκB in this nociceptive response (Fig. 2). Treatment with PDTC also diminished induction of TNF-α (Fig. 3A) and IL-1β (Fig. 3B) production by jararhagin. This finding suggests involvement of NFκB in the cytokine production in spinal cord induced by jararhagin.

3.4 Spinal glial activation following peripheral injection of jararhagin

Peripheral inflammation produces change that leads to central sensitization and activation of spinal cord glial cells. Next, we investigated whether peripheral stimulus with jararhagin induces microglia and astrocyte activation. The mice were inoculated with jararhagin (1 µg/paw 20µL) and times (1, 3, 5 and 7h) after the stimulus, animals were euthanized and the spinal tissue L4-L6 was dissected for RT-PCR. We observed an increase on mRNA expression of Iba-1 (Fig.4A) and GFAP (Fig.4B) 1 to 7h after stimulus.

3.5 Role of spinal glial in jararhagin-induced hyperalgesia

We observed activation of astrocyte and microglia, we next wonder whether jararhagin-induced pain depends on the activation of these cells through pharmacological blockage of them. The animals were pretreated with minocycline and α-aminoadipate 30 minutes before inoculation with jararhagin. The dose of minocycline (50 µg/ 5 µL i.t., Fig. 5A), and α-aminoadipate (100 nmol/ 5 µL i.t., Fig. 5B) reduced mechanical hyperalgesia 1-7 h after the injection of jararhagin stimulus (Fig.4). Moreover, pharmacological inhibition of these cells also decreased cytokine production 3 h after jararhagin injection in spinal cord (Fig.6). These results reported above suggest microglia and astrocyte play an important role in jararhagin-induced hyperalgesia.

4. Discussion

Jararhagin, a metalloproteinase from *Bothrops jararaca* snake venom, presents a zinc-dependent proteolytic, hemorrhagic and hyperalgesic activities, and correspond to 5–12% of *Bothrops jararaca* venom [18]. This study provides the first demonstration of a spinal cord nociceptive mechanism involving a glial cell activation with NFκB-dependent production of the hyperalgesic cytokines TNF-α and IL-1β.

NFκB is a key regulator of molecules and pathways important for inflammation and pain since it orchestrates the transcription of inflammatory genes such as TNF-α and IL-1β [51, 52]. TNF-α and IL-1β are major pro-inflammatory cytokines upregulated in the spinal cord under varied pain

conditions. Several evidences support an important role of TNF-α and IL-1β in pain sensitization and intrathecal treatments targeting these cytokines and NFκB have been shown to alleviate inflammatory, neuropathic, and cancer pain [53-56]. Selective inhibition of glial NFκB results in diminished neuropathic symptoms and reduces the expression of IL-6 and iNOS [56]. TNF-α and IL-1β can increase the transmission of pain signals by directly lowering the activation threshold of nociceptor sensory neurons [57, 58]. Corroborating the current understanding on NFκB, cytokines and pain, our findings demonstrated that peripheral injection of jararhagin induced TNF-α and IL-1β production in the spinal cord and intrathecal treatment with the NFκB inhibitor PDTC reduced TNF-α and IL-1β production. Furthermore, intrathecal administration of IL-1ra, etanercept, and PDTC reduced the mechanical hyperalgesia following jararhagin injection. Thus, these data demonstrate that the mechanical hyperalgesia induced by peripheral injection of jararhagin depends on spinal cord activation of NFκB and TNF-α and IL-1β production.

Considering that activation of microglia and astrocytes leads to activation the intracellular signalling via NFκB, this strongly indicates that neuroinflammation can promote inflammatory pain by releasing inflammatory mediators from activated glial cells [59, 60]. Therefore, considering the literature and our data on the contribution of spinal cord NFκB and cytokines in jararhagin-induced hyperalgesia, we postulated the involvement of glial cell in jararhagin effect. Our data showed that intrathecal treatment with glial cells inhibitors diminished mechanical hyperalgesia induced by jararhagin and these inhibitors attenuated TNF-α and IL-1β production in the spinal cord after jararhagin injection. Inhibiting microglia and astrocyte activation (minocycline and α-

aminoadipate, respectively) can prevent or delay neuropathic and inflammatory pain; indicating the importance of spinal microglia and astrocytes in the development of pain [61-64]. The intrathecal treatment with minocycline and α-aminoadipate inhibited mechanical hyperalgesia induced by jararhagin, thus, it is reasonable to interpret that the activation of glial cells by the peripheral input generated by jararhagin is responsible for the production of cytokines that are involved in pain development and maintenance.

Several studies demonstrated that changes in the levels of spinal Iba-1 (a marker for microglia) and GFAP (a marker for astrocytes) in the spinal cord can be used as indicators of elevated nociceptive states [65-68]. Our data clearly show jararhagin-induced the upregulation of Iba-1 and GFAP in spinal cord in mice. Microglia are the first cell type to activate in the CNS after peripheral injury, releasing pro-inflammatory mediators that activate astrocytes and neurons, which, in turn, maintain a long-term pathological state [69-72]. Our results demonstrated microglia and astrocyte activation started 1h after jararhagin injection. We propose that spinal microglia and astrocyte are crucial in the development of jararhagin induced mechanical hyperalgesia. However, the subtype of glia primarily responsible for TNF-α and IL-1β production during the hyperalgesia induced by jararhagin is still unknown. In this regard, co-localization study is necessary.

5. Conclusion

Our study demonstrated the involvement of spinal cells in mechanical hyperalgesia induced by jararhagin. The mechanism involves the production of pro-inflammatory cytokines in the spinal cord by NFκB.

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Conflict of interest

The authors declare no conflict of interest.

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Figure Captions

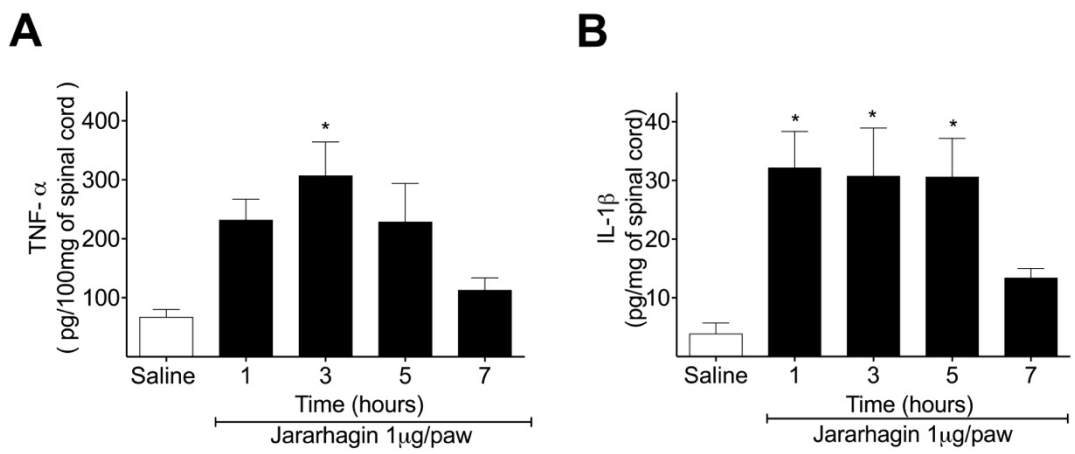

Figure 1. Peripheral jararhagin injection increases TNF-α and IL-1β levels in spinal cord

Mice received i.pl. injection of jararhagin (1 µg, 20 µL), and TNF- α (A) and IL-1β (B) production were evaluated in spinal tissue samples (L4-L6) 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 vs. saline group; #P<0.05 vs. jararhagin group (One-way ANOVA followed by Tukey's t test).

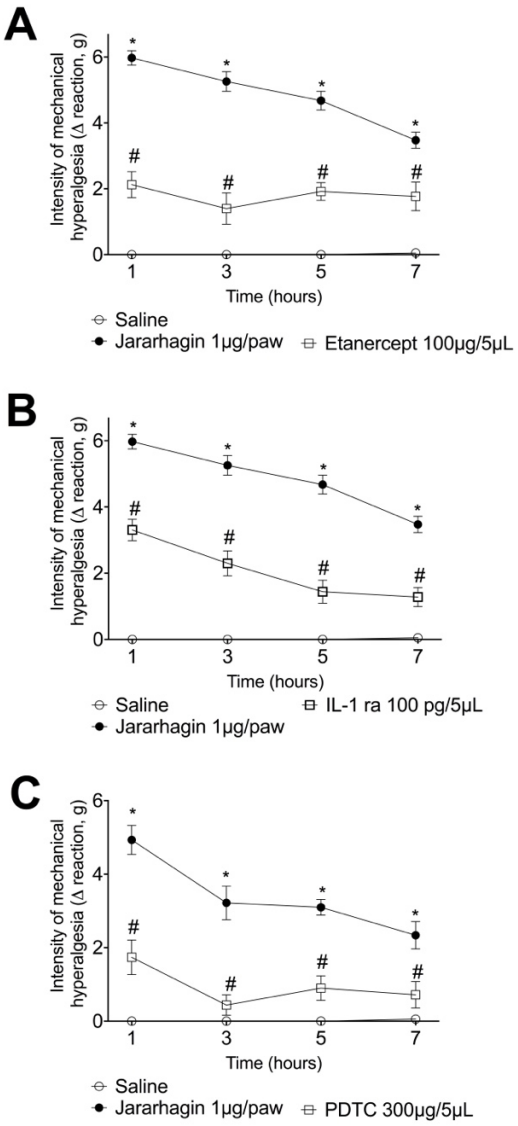

Figure 2. TNF-α, IL-1β, and NFkB inhibitors reduce jararhagin-induced mechanical hyperalgesia

Mice were treated with etanercept (100 µg/5 µL i.t.,) 30 min (A), IL-1ra (100 pg/5 µL i.t.,) 30 min (B) or PDTC (300 µg/5 µL i.t.) 30 min (C) before jararhagin (1 µg/paw) injection. Mechanical hyperalgesia was evaluated between 1-7 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 vs. saline group; #P<0.05 vs. jararhagin group (Two-way ANOVA followed by Tukey's t test).

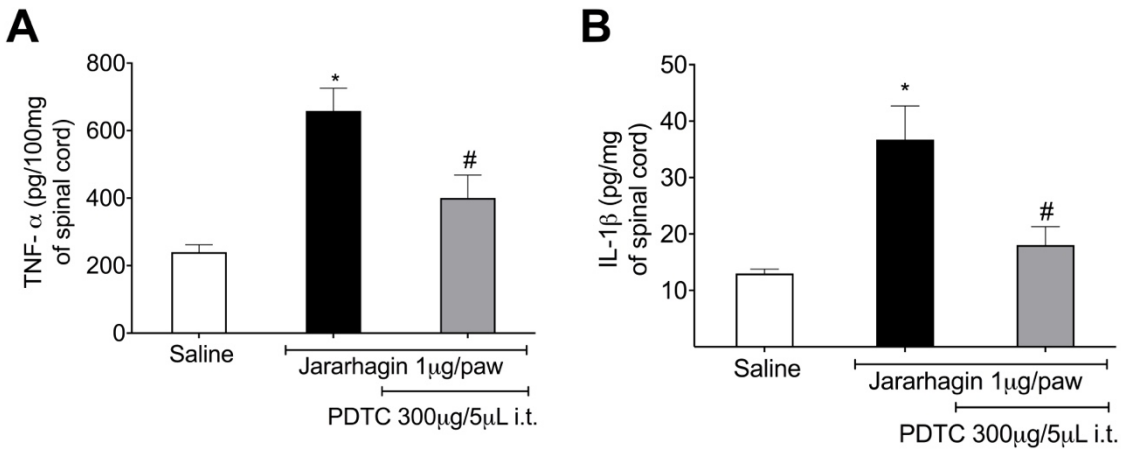

Figure 3. NFkB inhibitor decreases TNF-α and IL-1β production induced by jararhagin

Mice were treated with PDTC (300 μg/5 μL i.t.) 30 min before jararhagin (1 μg/paw) injection and TNF-α (A) and IL-1β (B) production were evaluated in spinal tissue samples (L4-L6) 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 vs. saline group; #P<0.05 vs. jararhagin group (One-way ANOVA followed by Tukey's t test).

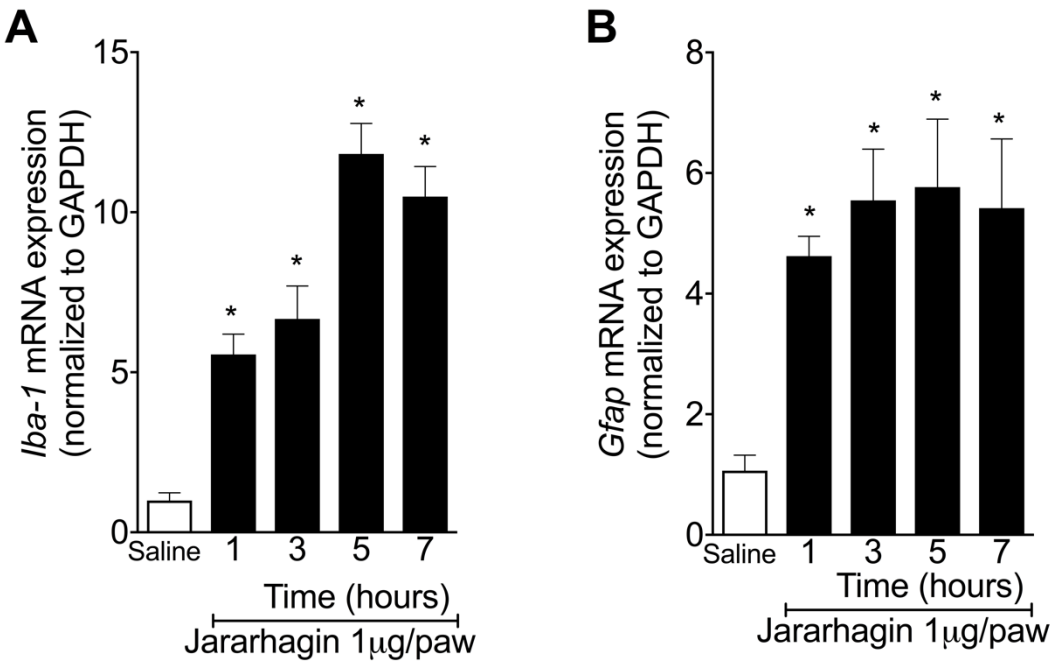

Figure 4. Jararhagin induces spinal cord up-regulation of Iba-1 and Gfap mRNA expression

Mice received i.pl. injection of jararhagin (1 µg, 20 µL), and Iba-1 (A) and Gfap (B) expression were evaluated by quantitative PCR in spinal tissue samples (L4-L6). Results are expressed as means ± SEM n = 6 mice per group, and are representative of 2 separated experiments. *P < 0.05 vs. saline control (ANOVA followed by Tukey's t test).

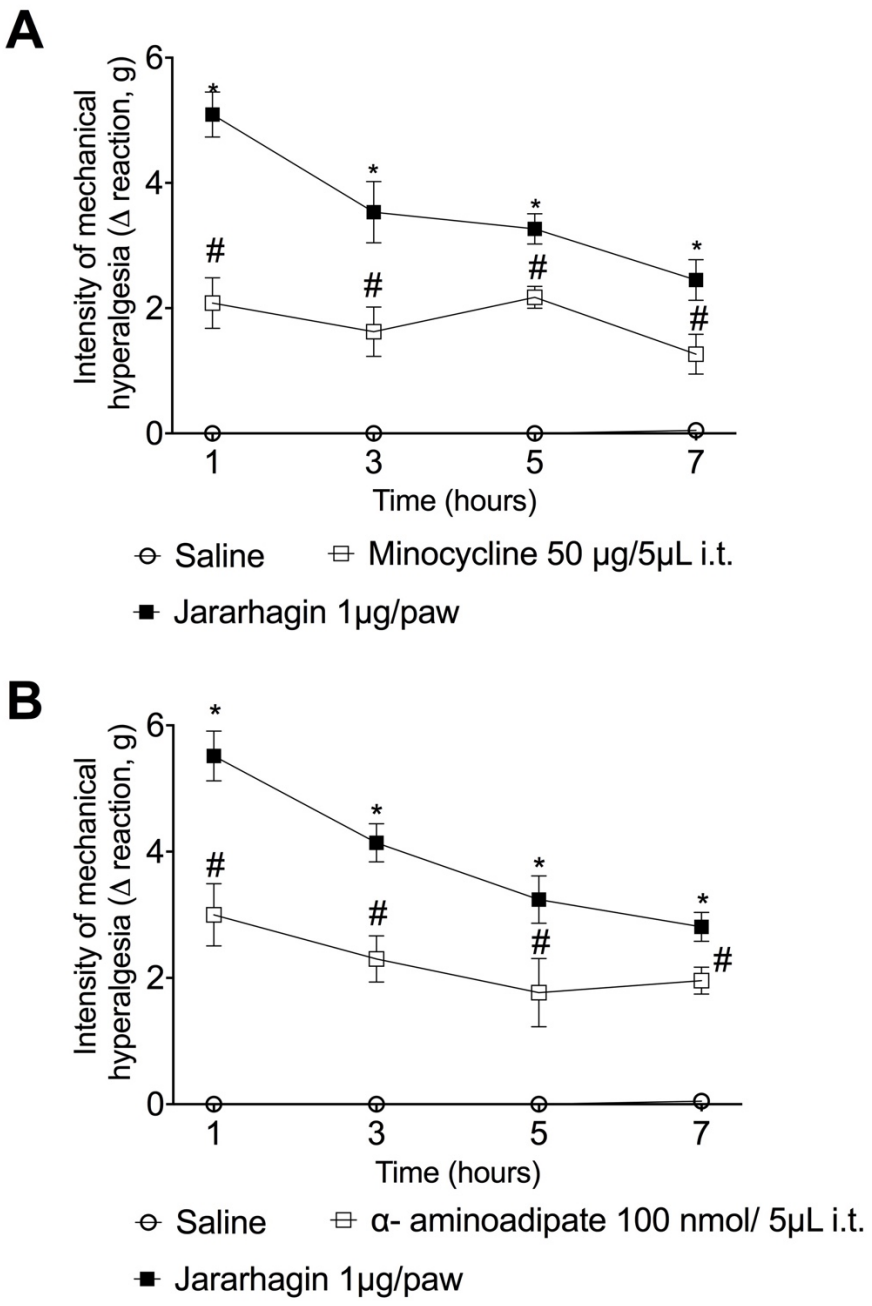

Figure 5. Role of glial cells in jararhagin-induced hyperalgesia.

The i.t. treatment with microglia inhibitor, minocycline (A) or α-aminoadipate (B), astrocytes inhibitor, inhibited the mechanical hyperalgesia induced by i.pl. administration jararhagin in the ipsilateral paw. Results are presented as means ± SEM. of 6 mice per group per experiment, and are representative of 2 separated experiments. *P<0.05 vs. saline group; #P<0.05 vs. jararhagin group (Two-way ANOVA followed by Tukey's t test).

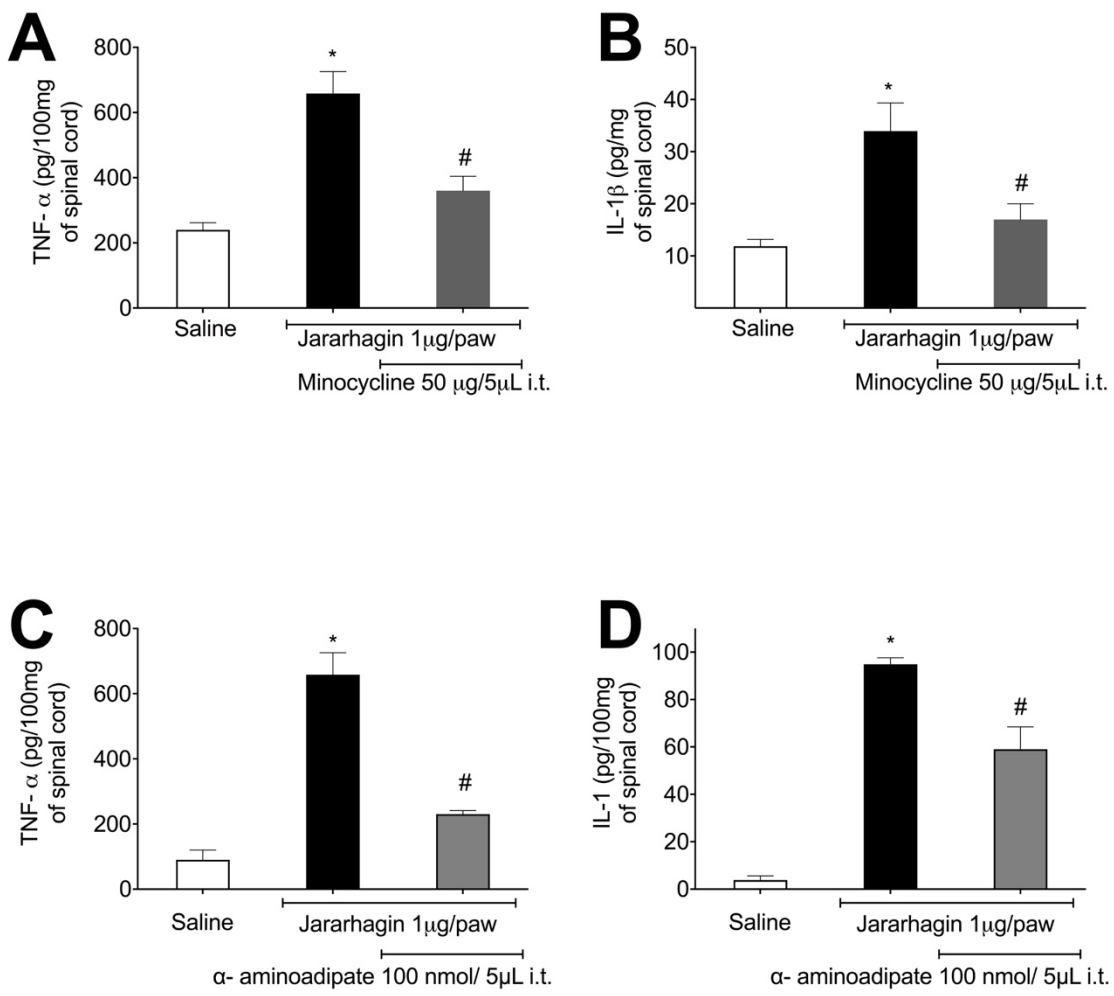

Figure 6. Microglia and astrocyte inhibitors decrease TNF-α and IL-1β production induced by jararhagin

Mice were treated with microglia inhibitor, minocycline (50 µg/ 5 µL i.t.) (A and B) or α- aminoadipate (C and D) (100 nmol/ 5 µL i.t.), astrocytes inhibitor, 30 min before jararhagin (1 µg/paw) injection and TNF- α and IL-1β production were evaluated in spinal tissue samples (L4-L6) 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 vs. saline group; #P<0.05 vs. jararhagin group (One-way ANOVA followed by Tukey's t test).

4.2 Jararhagin-induced mechanical hyperalgesia depends on spinal activation of MAP kinases in mice

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ABSTRACT

Peripheral stimulus with the metalloproteinase from *Bothrops jararaca* venom, jararhagin, induces spinal cord glial cell activation-dependent hyperalgesia. However, the role of the spinal MAP kinase in hyperalgesia induced by peripheral injection of jararhagin has not been investigated. Mice received intraplantar (i.pl.) injection of jararhagin and hyperalgesia and spinal cord MAP kinase activation were evaluated. Furthermore, jararhagin induced an increase of p-p38 and p-JNK in spinal cord. The effects of intrathecal (i.t.) injection of ERK inhibitor (PD 98059), p38 inhibitor (SB 202190) and JNK inhibitor (SP 600125) were investigated. Jararhagin induced significant mechanical hyperalgesia, which wasreduced by treatment with PD 98059, SB 202190 and SP 600125. These results provide better understanding of the nociceptive spinal cord mechanisms of jararhagin. Data support that spinal cord activation of MAP kinase pathways contribute to pain in snakebites and that targeting p38, ERK and JNK serves as pharmacological approach to reduce pain induced by snake venoms.

Keywords

ERK, JNK, p38, Metalloproteinase, *Bothrops jararaca* venom, Hyperalgesia, Pain

1 Introduction

The neglected tropical diseases constitute a group of diseases that afflict the world's poor and historically have not received as much attention as other diseases [1]. In 2017, the snake envenomation was considered a priority neglected tropical disease [1], World Health Organization (WHO) estimates that 2.7 million people each year develop clinical illness after snakebite, with morbidity and mortality affecting mostly individuals under 30 years old. In Brazil, the snakebites are considered an important public health problem and economic burden [2, 3]. In Brazil, the most relevant snake is *Bothrops jararaca,* whose venom is hemotoxic, myotoxic, coagulopathy diseases, cytotoxic, and causes severe pain [4].

In fact, pain induced by snakebite is a frequent clinical manifestation. Unfortunately, conventional therapies are not able to reverse this symptom [5]. The venom of *Bothrops jararaca* is a complex mixture of several classes of toxins including metalloproteases, serine proteinases, phospholipase A2, C-type lectins, bradykinin potentiating peptides, cysteine-rich proteins, L-amino acid oxidases, and snake venom vascular endothelial growth factor [6, 7]. The metalloproteinases are the major proteins of venom for pain in cases of *Bothrops jararaca* bites [8]. Jararhagin, a metalloproteinase from *Bothrops jararaca* venom*,* is capable of inducing pain [9, 10]. To date, few studies showed mediators and/or pathway activated by jararhagin to induce pain, and the spinal mechanism of jararhagin-induced hyperalgesia is still unknown.

Mitogen-activated protein kinases (MAP kinases) are important for intracellular signal transduction and play critical roles in regulating neural

plasticity, cellular proliferation and inflammation [11]. The MAP kinases family consists of three major members: extracellular signal-regulated kinases (ERK), p38, and c-Jun N-terminal kinase (JNK), which represent three separate signaling pathways [12]. Importantly, the inhibition of MAP kinases significantly attenuates inflammatory and neuropathic pain, which supports that targeting these signaling pathways is an analgesic approach [13-18].

Present data showed that jararhagin-induced peripheral hyperalgesia in mice depends on the activation of spinal p38, ERK, and JNK kinases. These results provide a better understanding of the hyperalgesic mechanisms of jararhagin and demonstrates that targeting MAP kinases can reduce pain triggered by jararhagin.

2. Methods

2.1. Animals and ethical statement

The experiments were performed on male swiss mice (20-25g, State University of Londrina, Londrina, PR, Brazil) housed in individually ventilated caging (IVC) rack for mice (AL22, Alesco®) with free access to food and water. All behavioral testing were performed between 9:00 am and 5:00 pm in a temperature-controlled room (24±2 ºC). Animals care and handling procedures were in accordance with the International Association for Study of Pain (IASP) guidelines and with the approval of State University of Londrina Ethics Committee on Animal Research and Welfare (CEUA number 2519.2015.83).

2.2. Jararhagin purification

The jararhagin was purified as previously described [10] in the Laboratory of Immunopathology, Butantan Institute.

2.4. Drugs

Drugs were obtained from the following sources: Jararhagin (1 µg/20 µL i.pl.) [10], PD 98059 (1, 3 and 10 µg/5 µl, intrathecal [i.t.]), SB 202190 (1, 3 and 10 µg/5 µl, i.t.) and SP 600125 (1, 3 and 10 µg/5 µl, i.t.) [14] were obtained from Sigma-Aldrich (St. Louis, MO, USA), 30 min before stimulus. Jararhagin was dissolved in saline (NaCl 0.9%) and all other compounds were dissolved in 20% DMSO in saline.

2.4 Experimental protocols

Mice received an intraplantar (i.pl.) injection of jararhagin (1 µg/paw) or saline and mechanical hyperalgesia was evaluated after 1, 3, 5, 7 h. Regarding the effect of pharmacological treatments over mechanical hyperalgesia mice were treated with PD 98059 (1, 3 and 10 µg/5 µl, i.t., 30 min before stimulus), SB 202190 (1, 3 and 10 µg/5 µl, i.t., 30 min before stimulus) and SP 600125 (1, 3 and 10 µg/5 µl, i.t., 30 min before stimulus). Doses of treatment were based on previous studies of our group and standardization in our laboratory [13-15]. Spinal MAP kinases activation were determined 1, 3, 5 and 7 h after jararhagin injection (1 µg/paw).

2.5 Electronic pressure–meter test for mice

Mechanical hyperalgesia was tested in mice as previously reported [19]. 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 was previously described [10].

2.8 Western blot assay

The L4–L6 segments of the spinal cord were dissected and homogenized in RIPA buffer containing protease and phosphatase inhibitors. The lysates were then homogenized and centrifuged (0.5 g for 10 min at 4°C). The protein extracts were separated by SDS-PAGE and transferred on to nitrocellulose membrane for the other analysis (GE Healthcare-Amersham, Pittsburgh, PA, USA). Membranes were then incubated in blocking buffer 5% bovine serum albumin or skim milk 5% for different times for each antibody at 4°C and incubated overnight at 4°C in the presence antibody phospho p38 (sc17852, 1:100) and total p38 (sc535, 1:100); phospho JNK (#4671, 1:500) and total JNK (#9252, 1:300), and β-actin (1:10.000, Sigma-Aldrich) was used for loading controls. After washing in TBS with Tween 20, the membrane was incubated with secondary antibody for 2 h at room temperature. Protein was visualized by chemiluminescence with ECL detection reagent (GE Healthcare-Amersham). The molecular mass of protein was confirmed by Precision Plus Protein Standards (Bio-Rad, Hercules, CA, USA).

2.10 Statistical analysis

Results are presented as means ± SEM of measurements made on 6 mice in each group per experiment, representative of 2 independent experiments.

Two-way repeated measure analysis of variance (ANOVA) followed by Tukey's post hoc was used when responses were measured at different times points after the stimulus injection. Statistical differences were considered to be significant at $P< 0.05$ in the software Prism 6.0.

3. Results

Peripheral jararhagin injection activated p38 *and JNK in spinal cord*

Many peripheral stimuli are capable to induced MAP kinase activation in spinal cord, thus we first evaluated whether jararhagin was capable to activated MAP kinases pathway in spinal cord in mice. The animals received jararhagin (1 µg/paw) or saline i.pl. injection and after 1, 3, 5, and 7 h the spinal cord (L4-L6) were collected and the p38 and JNK activation were determined by Western blot. Jararhagin induced significantly phosphorylation of MAP kinases in different times in spinal cord (Fig. 1). Jararhagin increased the phosphorylated JNK (Fig. 1A) at 5 and 7 h and the phosphorylated p38 at 1, 3, 5, and 7 h (Fig. 1B).

Role of spinal ERK, p38 and JNK in jararhagin-induced hyperalgesia

We observed an increase on MAP kinase activation we next investigated whether jararahagin-induced pain depends on MAP kinases phosphorylation. Mice were treated i.t. with specific EKR (PD 98059; Fig 2), p38 (SB 202190; Fig.3), JNK (SP 600125; Fig. 4) inhibitor or vehicle (5 µl of 20% DMSO in saline) 30 min before jararhagin stimulus (1 µg/paw). We evaluated mechanical hyperalgesia at 1, 3, 5 and 7 h after jararhagin injection (Fig. 3). The PD 98059 (Fig. 2) and SP 60012

(Fig. 4) doses of 3 and 10 µg inhibited jararhagin-induced hyperalgesia at all time points, and the SB 202190 (Fig. 3) doses of 3 and 10 µg inhibited hyperalgesia at 3, 5, 7 time points. These findings suggest that MAP kinases pathway can contribute to enhanced jararhagin-induced hyperalgesia.

4. Discussion

Metalloproteases are the major components of Viper venoms [20, 21]. The most medically important viper snake in Latin America is *Bothrops jararaca*. The first metalloprotease isolated from *Bothrops jararaca* venom was jararhagin [21, 22]. Jararhagin is responsible for many systemic and local effects of snake envenomation, as anticoagulant, hemorrhagic, platelet aggregation inhibition, inflammation, and pain [21]. Jararhagin induces hyperalgesia in mice [10] and rats [9]. This mechanical hyperalgesia depends on TNF-α and IL-1β production and NFκB activation in peripheral tissues [10] as well as on glial cells, cytokines (TNF-α and IL-1β) and transcription nuclear factor NFkB in the spinal cord (Ferraz et al., unpublished). Nevertheless, few studies addressed the mechanisms involved in pain produced jararhagin. In this study, we identified an essential role of spinal cord MAP kinase signaling pathways in the induction of hyperalgesia by peripheral administration of jararhagin.

MAP kinases are crucial in intracellular signaling pathways that modulate the nociceptive responses [11]. MAP kinases play a key role in production of CNS inflammatory cytokines, free radicals [23, 24] and activation of NFκB [25]. TNF-α and IL-1β play an essential role in central sensitization and pain [26, 27] and recent evidence demonstrated jararhagin increased TNF-α and IL-1β production

in spinal cord was depend on NFκB phosphorylation (Ferraz *et al*., unpublished). The biosynthesis of TNF-α and IL-1β and NFκB, as well as many other inflammatory mediators are up-regulated by p38 and ERK [23, 24, 28]. After activation, p38 increases the synthesis of several pro-inflammatory mediators such as COX-2 [16], IL-1β [29] and iNOS [30] in the spinal cord possibly via transcriptional regulation. Importantly, pre-treatment with the ERK inhibitor PD98059 prevented increases in p65 translocation, NFκB luciferase activity, and phospho-IKKα/β suggesting that NFκB activation involves ERK activation [31]. JNK pathway also was involved in the expression of TNF-α in rheumatoid arthritis [32].

The present data demonstrate that jararhagin is capable of inducing mechanical hyperalgesia dependent on spinal cord MAP kinase phosphorylation. Our findings and several studies show targeting JNK, ERK, and p38 provide effective relief of pain [13-15]. Ferraz et al., (unpublished) demonstrated hyperalgesia induced by jararhagin was mediated by spinal cord TNF-α and IL-1β, thus our data suggest that inhibiting MAP kinase activation decreases TNF-α and IL-1β production in the spinal cord and diminishes jararhagin-induced hyperalgesia.

Recent studies demonstrated MAP kinase activation in microglia [13, 33-35] and astrocyte [11, 36, 37]. This process promotes intracellular events that contribute to the peripheral and central sensitization that are manifested at the behavioral and cellular levels [38, 39]. MAP kinases exhibit distinct activation patterns in glial cells after painful injuries [11, 40]. Numerous studies have shown increased phosphorylation of p38 in spinal cord microglia after nerve injury [41, 42], formalin-induced acute inflammatory pain [16], and postoperative pain [43].

JNK activation in spinal astrocytes after nerve injury [44], CFA-induced persistent inflammatory pain [45] and bone cancer [46]. Differently, ERK phosphorylation is highly dynamic. There is ERK activation in microglia in the early-phases and gradually transition to astrocytes in the late phase [36, 47, 48]. An earlier study demonstrated spinal astrocyte and microglia activation after jararhagin injection in mice (Ferraz et al., unpublished) and phospholipase A2 from *Bothrops asper* venom was able to activated spinal microglia and astrocytes after peripheral injection [49]. Our data and these studies suggest that MAP kinases were activated in the spinal cord and this pathway activates transcription of pro-inflammatory cytokines after jararhagin injection. This hypothesis is further supported by the findings that intrathecal administration of selective p38, JNK and ERK inhibitor attenuated mechanical hyperalgesia induced by jararhagin. Nevertheless, co-localization studies are necessary to consolidate the present behavioural and pharmacological data. In fact, these results demonstrate that targeting p38, ERK and JNK could be great pharmacologic target to control pain induced by snake venoms.

5. Conclusion

In conclusion, the present study demonstrated that spinal cord MAP kinases ERK, p38, JNK play an important role in hyperalgesia induced by jararhagin. These findings contribute to the development of better snakebite therapies.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

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Figure Captions

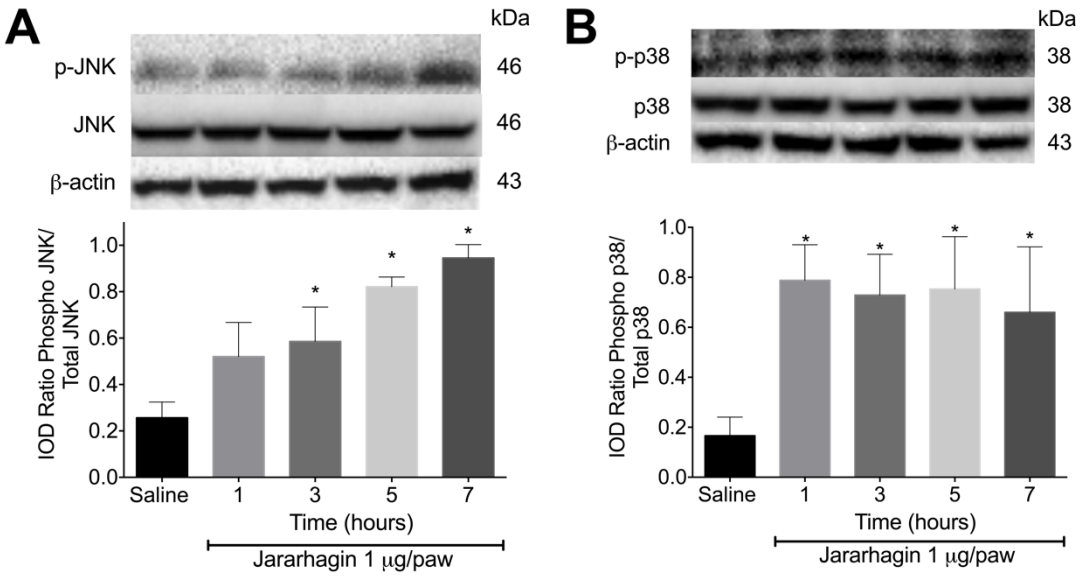

Figure 1: Jararhagin-induced activation of p38 and JNK in spinal cord.

Mice received i.pl. injection of jararhagin (1 µg, 20 µL), and total and phospho p38 (A) and total and phospho JNK (B) were evaluated by western blot in spinal tissue samples (L4-L6). Results are expressed as means ± SEM n = 6 mice per group *P < 0.05 vs. saline control. (ANOVA followed by Tukey's t test).

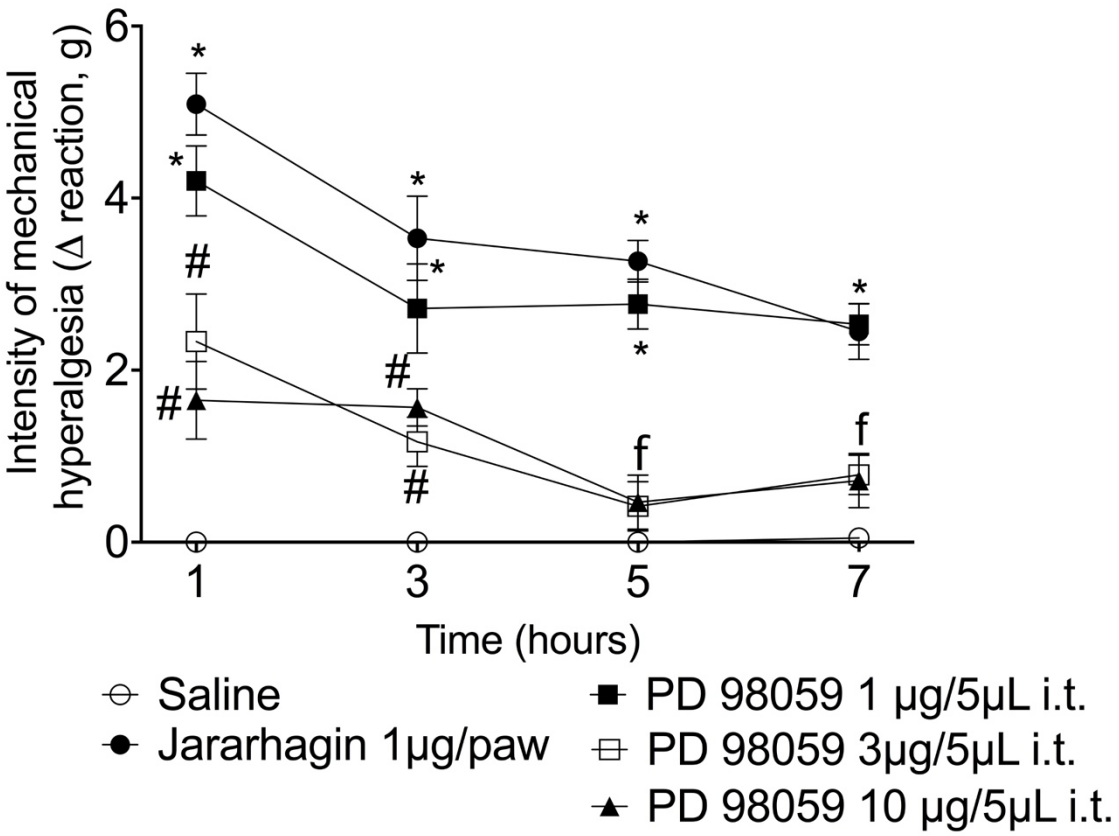

Figure 2: ERK inhibitor reduces jararhagin-induced mechanical hyperalgesia.

Mice were treated with PD 98059 (1, 3 or 10 µg/5 µL i.t.,) 30 min (A) before jararhagin (1 µg/paw) injection. Mechanical hyperalgesia was evaluated between 1-7 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 vs. saline group and 3 and 10 µg dose; #P<0.05 vs. jararhagin and saline group; *f*P<0.05 vs. jararhagin group (Two-way ANOVA followed by Tukey's t test).

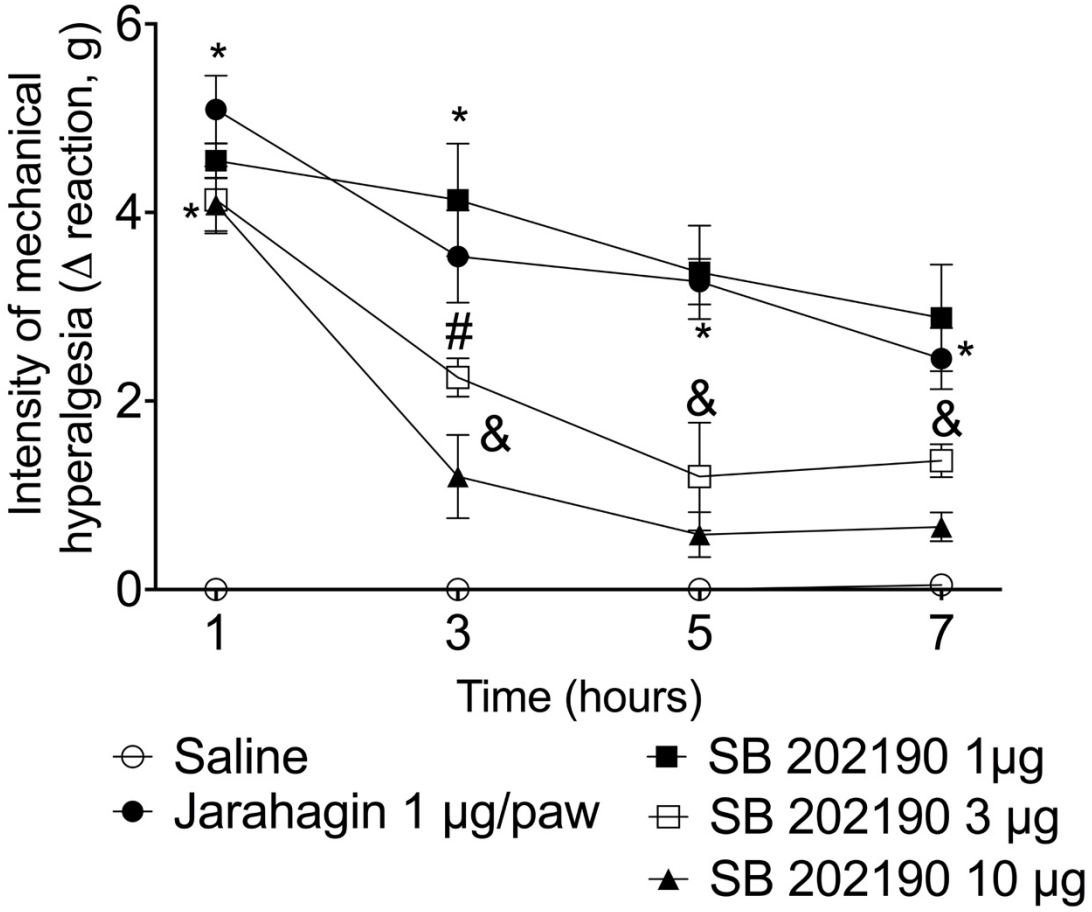

Figure 3: p38 inhibitor reduces jararhagin-induced mechanical hyperalgesia.

Mice were treated with SB 202190 (1, 3 or 10 µg/5 µL i.t.,) 30 min (A) before jararhagin (1 µg/paw) injection. Mechanical hyperalgesia was evaluated between 1-7 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 vs. saline group; #P<0.05 vs. jararhagin, saline, 1 and 10 group; &P<0.05 vs. jararhagin and saline group (Two-way ANOVA followed by Tukey's t test).

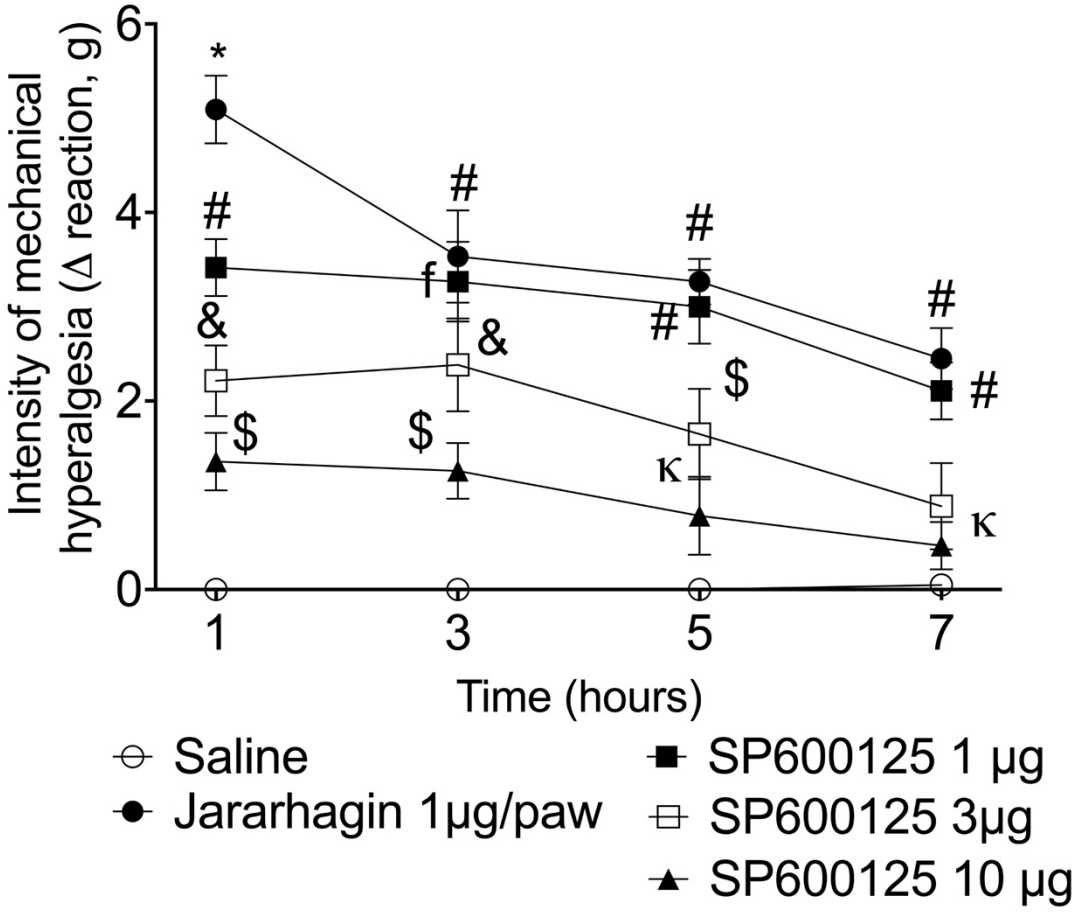

Figure 4: JNK inhibitor reduces jararhagin-induced mechanical hyperalgesia.

Mice were treated with SP 600125 (1, 3, or 10 µg/5 µL i.t.,) 30 min (A) before jararhagin (1 µg/paw) injection. Mechanical hyperalgesia was evaluated between 1-7 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 vs. saline, 1, 3, and 10 group; #P<0.05 vs. saline, 3, and 10 group; &P<0.05 vs. jararhagin, saline and 10 group; *f*P<0.05 vs. saline and 10 group; $P<0.05 vs. saline and jararhagin group; *k*P<0.05 vs. jararhagin group (Two-way ANOVA followed by Tukey's t test).

4.3 Peripheral mechanisms involved in the nociception triggered by *Tityus bahiensis* and *Tityus serrulatus* venom

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ABSTRACT

Scorpionism is a public health burden in Brazil. *Tityus bahiensis* and *Tityus serrulatus* are responsible for the majority of accidents in the Southeastern region of Brazil. The hyperalgesic mechanisms of *Tityus bahiensis* and *Tityus serrulatus* venom were investigated focusing on the role of recruitment of cells, proinflammatory cytokines (TNF-α and IL-1β) and the transcription factor NFκB. Intraplantar administration of *Tityus bahiensis* and *Tityus serrulatus* venom 0.2, 0.6, 1.2 and 2.4μg/20 μL i.pl. induced mechanical and thermal hyperalgesia, and increased MPO and NAG activity at 5h after scorpion venoms injection in the paw tissue. *Tityus bahiensis* and *Tityus serrulatus* venom (2.4 μg) induced overt pain-like behaviour and increased TNF-α levels at and IL-1β levels after scorpion venoms injection in the paw tissue. The systemic pre-treatment with etanercept (10 mg/kg), IL-1ra (30 mg/kg) and PDTC (NFκB inhibitor; 100 mg/kg) inhibited *Tityus bahiensis* and *Tityus serrulatus* venom-induced mechanical and thermal hyperalgesia, MPO and NAG activity and overt pain-like behaviour. Thus, these data demonstrate the involvement of neutrophils, macrophages, pro-inflammatory cytokines TNF-α and IL-1β and nuclear transcription factor NFκB in *Tityus bahiensis* and *Tityus serrulatus* venom-induced mechanical thermal hyperalgesia and overt pain-like behaviour indicating that targeting these mechanisms might contribute to reduce the pain induced by scorpion venoms.

Keywords: Cytokines; Hyperalgesia; *Tityus bahiensis*; *Tityus serrulatus* NFκB

1 Introduction

Scorpionims is a public health burden in the worldwide [1]. In 2017, approximately 125.000 scorpionism were reported in Brazil [1, 2]. The majority of these scorpion envenomation were associated with the *Tityus* genus, particularly the species *Tityus bahiensis* and *Tityus serrulatus* [3, 4]. Clinical manifestations of *Tityus bahiensis* and *Tityus serrulatus* sting include vomiting, tachycardia, bradycardia, tremors, respiratory failure, psychomotor agitation, salivation, lachrymation, increased gastrointestinal mobility, paraesthesia and pain [5, 6].

So far, the majority of the studies investigating *Tityus bahiensis and Tityus serrulatus* venom focus on understanding the systemic effects such as neurotoxicity [7] and respiratory failure[8]. However, the mechanisms underlying the inflammatory and nociceptive effects of *Tityus bahiensis and Tityus serrulatus* crude venom or its isolated molecules are poorly understood. *Tityus bahiensis* venom induces oedema [9] and leukocyte recruitment in rats [8] , and Tb II-I, a protein isolated from *Tityus bahiensis* venom, increased levels of IL-6 and TNF-α in rats [10]. Injection of *Tityus serrulatus* venom into the paw of rats causes a dose-dependent paw oedema, mechanical hyperalgesia and flinches dose-dependently due to the increase of eicosanoids, histamine and 5-hydroxytryptamine [6, 11]. Ts8, a peptide isolated from *Tityus serrulatus* venom, modulates Kv4.2 channels inducing overt pain-like behaviour (licking and flinches) and mechanical hyperalgesia [12]. Recruitment of neutrophils and macrophages, cytokines production and activation of NFkB have been shown to play a key role in hyperalgesia and overt pain-like behaviour in several animal models of pain. The activation of resident inflammatory cells and receptors

triggers activation of NFκB, which promotes the transcription of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, IL-8) [13-18]. For instance, TNF-α induces the production of IL-6 and IL-1β and stimulates the activation of cyclooxygenase, inducing prostanoid production and inflammatory hyperalgesia [19, 20].

In this study, we demonstrated that pro-inflammatory cytokines and NFkB induce a major role in the pain induced by *Tityus bahiensis and Tityus serrulatus* venom. Our data show that *Tityus bahiensis and Tityus serrulatus venom*-induced hyperalgesia and overt pain-like behaviour in mice were depend on TNF-α and IL-1β and transcription nuclear factor NFkB. These results provide a better understanding of the peripheral nociceptive mechanism induced by scorpion venoms.

2. Methods

2.1. Animals

The experiments were performed on male Swiss mice (25 g) from Londrina State University, Londrina, PR, Brazil. Mice were housed in standard clear plastic cages with food and water *ad libitum* and water and temperature of 23 °C ± 2. A 12/12 h light/dark cycle was used with lights on at 6 a.m and off at 6 p.m. Animal care and handling procedures were in accordance with the International Association for Study of Pain (IASP) guidelines, and approved by the Ethics Committee of the Londrina State University (CEUA Nº 21366.2015.72).

2.2. Tityus bahiensis and *Tityus serrulatus venom*

Tityus bahiensis and *Tityus serrulatus* lyophilized crude venom supplied by Butantan Institute (São Paulo, Brazil) and kept at −20 °C.

2.3 Drugs

Drugs used were: *Tityus bahiensis* and *Tityus serrulatus* venom (0.2, 0.6, 1.2 and 2.4µg/20 µL i.pl.) [9],etanercept (10 mg/kg, 200 µL, i.p., 48 h plus 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., 30 min before stimulus) from NIBSC (National Institute of Biological Standards and Control, UK); pyrrolidine dithiocarbamate (PDTC) (100 mg/kg, 100 µL s.c., 30 min before stimulus) [13]. *Tityus bahiensis* and *Tityus serrulatus* venom, etanercept and IL1-ra were dissolved in saline, and PDTC was dissolved in 2% DMSO in saline.

2.4 Experimental protocols

Mice received an intraplantar (i.pl.) injection of *Tityus bahiensis* and *Tityus serrulatus* venom (0.2, 0.6, 1.2 and 2.4µg/20 µL i.pl.) or saline and mechanical and thermal hyperalgesia was evaluated after 1, 3 and 5h. Overt pain-like behavior was evaluated for 30 min after *Tityus bahiensis* or *Tityus serrulatus* venom injection (2.4 µg/paw). Regarding the effect of pharmacological treatments over mechanical and thermal hyperalgesia, and overt pain-like behavior mice were treated etanercept (10 mg/kg, 200 µL, i.p., 48 h plus 1 h before stimulus);

IL-1ra (30 mg/kg, 200 µL, i.p; PDTC (100 mg/kg, 100 µL s.c.) or saline. Cytokine (TNF-α and IL-1β) levels were determined 1, 3 and 5 h after *Tityus bahiensis* and *Tityus serrulatus* venom injection (2.4 µg/paw) and MPO and NAG activity were determined at 5 h after *Tityus bahiensis* and *Tityus serrulatus* venom injection. Doses of treatment were based on previous studies of our group and standardization in our laboratory [13].

Mechanical hyperalgesia test

Mechanical hyperalgesia was tested in mice by the electronic von Frey anesthesiometer: Insight, Ribeirão Preto, SP, Brazil adapted with a 0.5 mm^2 contact area polypropylene tip as previously reported [21]. The results are expressed by delta (∆) withdrawal threshold (in grams), which was calculated by subtracting the zero-time mean measurements from the mean measurements after stimulus at the indicated time points.

Thermal hyperalgesia test

Thermal hyperalgesia was tested in mice by hot plate (IITC Life Science, Inc., Woodland Hills, CA, USA) maintained at 55°C±1ºC as described [22, 23]. A cut-off of 20 seconds was set to avoid tissue damage [24].

Overt pain-like behaviour test

The number of paw flinches and the time spent licking the paw were determined between 0 and 30 min after i.pl. injection of *Tityus bahiensis* and *Tityus serrulatus* venom as previously reported [24].

Myeloperoxidase (MPO) and N-acetylglucosaminidase activity (NAG) assays

Myeloperoxidase and N-acetylglucosaminidase assays were performed as indirect indicators of neutrophil and macrophage recruitment, respectively. Paw skin samples were collected 5 h after *Tityus bahiensis* and *Tityus serrulatus* venom injection and homogenized. The supernatants were used for both assays. The myeloperoxidase and N-acetylglucosaminidase activity assay was performed as previously described [25, 26],

Cytokine measurement

Paw skin samples were collected 0.5, 1 and 3 h after the injection of *Tityus bahiensis* and *Tityus serrulatus* venom and homogenized. The supernatants used to measure IL-1β and TNF-α levels using commercial ELISA kits according to the manufacturer's instructions (eBioscience®, Ready-SET-Go) as previously reported [13].

2.10 Statistical analysis

Results are presented as means ± SEM of measurements made on 6 mice in each group per experiment, representative of 2 independent experiments. Two-way repeated measure analysis of variance (ANOVA) followed by Tukey's post hoc was used when responses were measured at different times points after the stimulus injection. Statistical differences were considered to be significant at $P < 0.05$ in the software Prism 6.0.

3. Results

Tityus bahiensis and *Tityus serrulatus* venom *induced dose-dependent mechanical hyperalgesia, thermal hyperalgesia, MPO and NAG activity*

Firstly, we evaluated the efficacy of *Tityus bahiensis* and *Tityus serrulatus* venom (0.2, 0.6, 1.2 and 2.4µg/20 µL i.pl.) induced mechanical hyperalgesia and thermal hyperalgesia. *Tityus bahiensis* (Fig. 1A and 1B) and *Tityus serrulatus* (Fig. 1C and 1D) venom induced dose-dependent mechanical hyperalgesia and thermal hyperalgesia. Next, we investigated whether injection of venoms induce MPO and NAG activity. All doses of *Tityus bahiensis* (Fig. 2A and 2B) and *Tityus serrulatus* (Fig. 2C and 2D) venom induced significantly MPO and NAG activity. The hyperalgesic response of 2.4 µg/paw of *Tityus bahiensis* and *Tityus serrulatus* venom was significantly higher than the lower doses, thus, it was selected for the following experiments.

Peripheral Tityus bahiensis and *Tityus serrulatus* venom *injection increased TNF-α and IL-1β levels*

Next, we investigated whether peripheral injection of venoms induce cytokine production. *Tityus bahiensis* venom increased TNF-α production at 1 and 3 h (Fig. 3A) and IL-1β production at 0.5, 1 and 3 h (Fig. 3B) and *Tityus serrulatus* venom induced TNF-α and IL-1β production at 1 and 3 h (Fig. 3C and 3D).

Tityus bahiensis and *Tityus serrulatus* venom *induced overt pain-like behaviour*

Next, we sought to evaluate of scorpion venoms causing spontaneous pain. *Tityus bahiensis* (Fig. 4A and 4B) and *Tityus serrulatus* (Fig. 4C and 4D) venom *(*2.4 µg/paw) induced increase in the number of paw flinches and time spent licking the paw.

Target TNF-α, IL-1β and NFkB reduce mechanical hyperalgesia, thermal hyperalgesia, and MPO and NAG activity induced by Tityus bahiensis and *Tityus serrulatus* venom

We investigated if Etanercept, IL-1ra and PDTC treatment inhibited mechanical hyperalgesia, thermal hyperalgesia, and MPO and NAG activity induced by *Tityus bahiensis* and *Tityus serrulatus* venom. Etanercept, IL-1ra and PDTC treatment inhibited *Tityus bahiensis* (Fig. 5) and *Tityus serrulatus* (Fig. 6) venom-induced mechanical and thermal hyperalgesia at all time points evaluated. Etanercept, IL-1ra and PDTC reduced *Tityus bahiensis* (Fig. 7). and *Tityus serrulatus* (Fig. 8). venom-induced MPO and NAG activity.

Inhibitors of TNF-α, IL-1β and NFkB diminish overt pain-like behaviour induced by Tityus bahiensis and *Tityus serrulatus* venom

Finally, we evaluated the effect of inhibitors of TNF-α, IL-1β and NFkb scorpion venoms-induced overt pain-like behaviour. Treatment with etanercept, IL-1ra and PDTC reduced scorpion venoms-induced flinches and time spent licking the paw (Fig. 9 and 10).

4. Discussion

Tityus bahiensis and *Tityus serrulatus* sting are the most important form of envenomation by scorpion in Brazil. Local pain is the main clinical manifestation of these scorpion stings, and the mechanisms involved in pain manifestation are still not well described in the literature. In the present manuscript, we studied the mechanisms through which *Tityus bahiensis* and *Tityus serrulatus venom* produce spontaneous nociception and hyperalgesia in mice by using different pharmacological tools through behavioural tests. Our results showed that a single intraplantar injection of *Tityus bahiensis* or *Tityus serrulatus* venom in mice produced extended nociception that was mediated by TNF-α, IL-1β and NFkB.

Recruited neutrophils and macrophages play an essential role in inflammatory pain by producing nociceptive mediators, such as TNF-α and IL-1β. A well-recognized role of NFκB is regulation of inflammatory responses [27]. The pro-inflammatory function of NFkB has been extensively studied in macrophages. For instance, in response to diverse PAMPs and DAMPs, macrophages become rapidly activated and secrete a large array of cytokines and chemokines [27, 28]. The macrophages are capable of differentiating into phenotypically distinct states, including the classically activated (M1) and the alternatively activated (M2) macrophages. NFκB is a key transcription factor of M1 macrophages and is required for the induction of a large number of inflammatory genes, including those encoding TNF-α, IL-1β, IL-6, and cyclooxygenase-2. TNF-α induces the production of inflammatory mediators such as IL-1β and PGE2, in turn, act on nociceptive neurons and increase hyperalgesia [29]. In this context, we observed that mice treated with etanercept, IL-1ra or PDTC presented diminished

hyperalgesia and MPO and NAG activity induced by scorpion venoms in inflammation site when compared to the control group. Thus, it is reasonable to interpret that recruitment of cells by the peripheral input generated by scorpion venom is responsible for the production of pro-inflammatory cytokines that are involved in sensitization of primary nociceptive neurons and in pain maintenance.

In the inflammatory pain context, *Tityus bahiensis* and *Tityus serrulatus* venom induced overt pain-like behaviour in mice. Paw flinching is considered a peripheral and spinal response and paw licking presents the structural mechanisms of flinching plus supraspinal nociceptive structures [30, 31]. Indeed, it was observed that *Tityus bahiensis* and *Tityus serrulatus* venoms can directly activate nociceptors provoking an instant flinching and licking behavior in injected animals. Recently, it has been shown that different toxins of scorpion venom can modulate TRP channels, Nav channels and Kv channels directly. This suggest that compounds of these venoms can activate or bind to channels existing on nociceptors membrane and in a second phase, based on the description of formalin test, stimulate pro-inflammatory mediators' production. Therefore, it is likely that scorpion venoms activate peripheral, spinal and supraspinal responses which are amenable to etanercept, IL-1ra and PDTC treatment by reducing pro-inflammatory mediators.

While some venoms are of relatively simple composition, many contain hundreds to thousands of individual components with distinct pharmacological activity [32]. Scorpion venom is a mixture of peptides, including, bradykinin-potentiating and anionic peptides and small to medium proteins, such as ion channel toxins, metalloproteinases and phospholipases that together cause severe clinical manifestations [33]. The major toxins identified in *Tityus bahiensis*

and *Tityus serrulatus* venom are potassium and sodium channel toxins, whereas metalloproteinases showed high abundance in theses venoms. [33, 34]. Recent studies have demonstrated that metalloproteinases and phospholipases from animal venoms exert potential hyperalgesic effects in mice by modulating pro-inflammatory cytokines, PGE2 and LTB4, nitric oxide, NFkB, and recruitment of leukocytes [13, 35-37]. Thus, these findings suggest that metalloproteinases and/or phospholipases or both can play a crucial role in the development and maintenance of scorpion venom-induced hyperalgesia. However, other studies with protein isolated from these venoms are necessary.

5. Conclusion

In conclusion, the present study demonstrated that recruitment of neutrophils and macrophages, NFkB activation and TNF-α and IL-1β production play an important role in hyperalgesia induced by *Tityus bahiensis* and *Tityus serrulatus* venom. These findings contribute to the development of better scorpionism therapies.

Conflict of interest

The authors declare no conflict of interest.

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Figure Captions

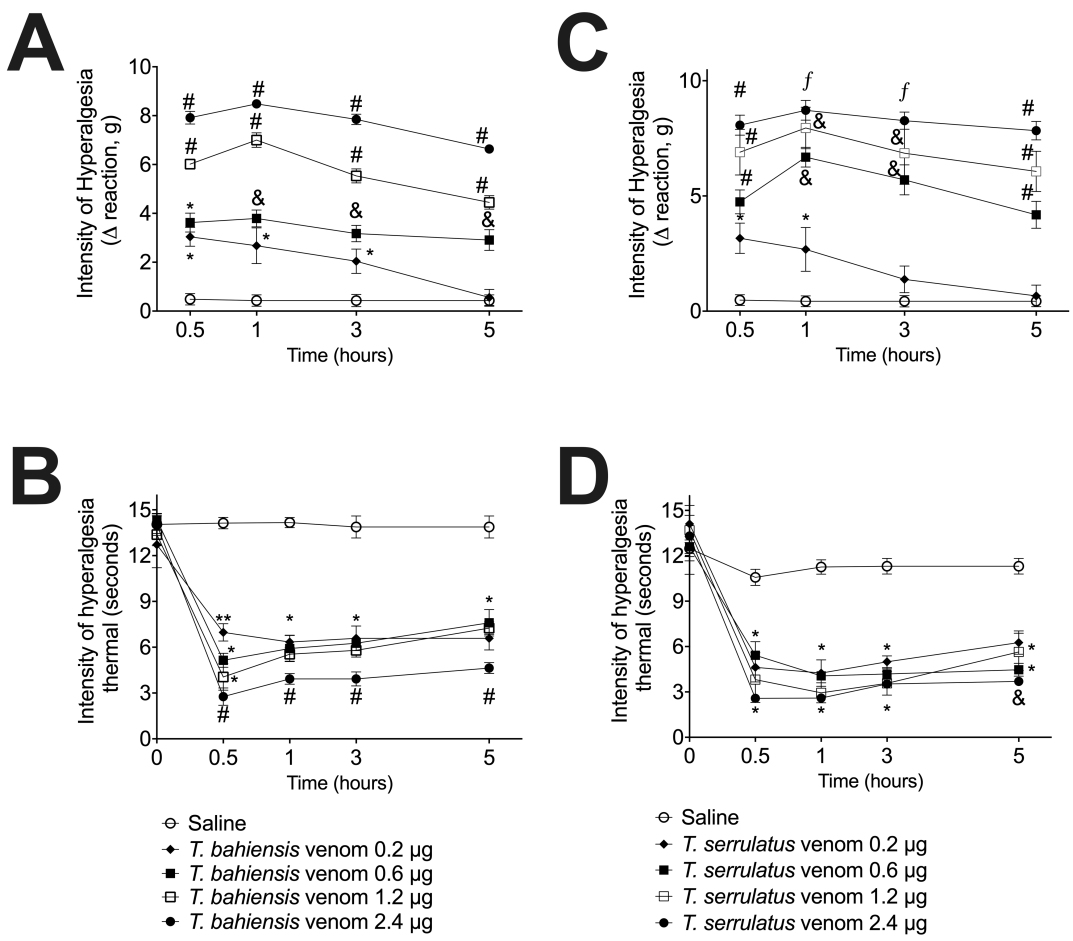

Figure 1. *Tityus bahiensis (*A and B) and *Tityus serrulatus* (C and D) venom induce dose-dependent mechanical hyperalgesia and thermal hyperalgesia. Mice received *Tityus bahiensis* (panel A and B) or *Tityus serrulatus* (panel C and D) venom via i.pl. 0.2, 0.6, 1.2 or 2.4µg/paw or saline (20 µL). Mechanical and thermal hyperalgesia were evaluated after 0.5-5h. Results are presented as means ± s.e.m. of 6 mice per group per experiment, and are representative of 2 separated experiments. *P<0.05 compared to the saline group; #P<0.05 compared to the group (Two-way ANOVA followed by Tukey's t test).

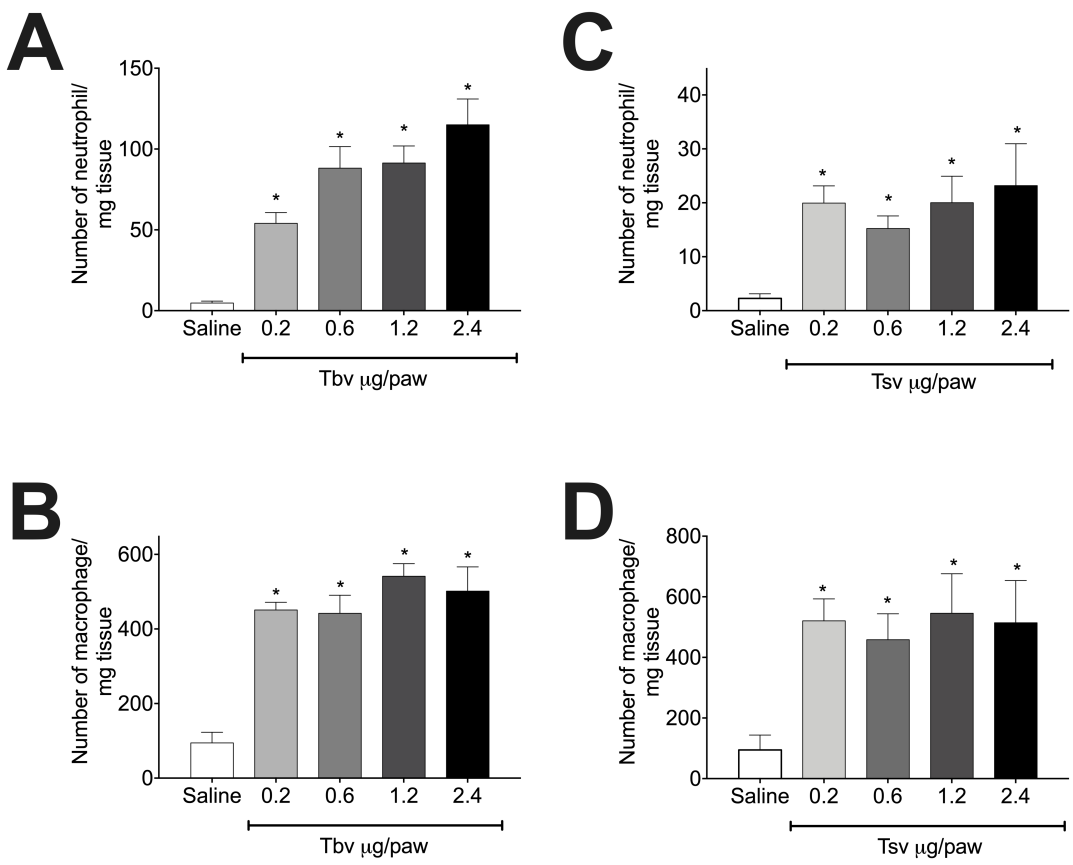

Figure 2. *Tityus bahiensis* (A and B) and *Tityus serrulatus* (C and D) venom induce recruitment of neutrophils and macrophages in the mice paw skin. Mice received *Tityus bahiensis* (panel A and B) or *Tityus serrulatus* (panel C and D) venom via i.pl. 0.2, 0.6, 1.2 or 2.4μg/paw or saline (20 μL). Paw skin samples were collected at 5h after stimulus with scorpion venoms, and MPO and NAG activity were measured. Results are presented as means ± s.e.m. of 6 mice per group per experiment, and are representative of 2 separated experiments. *P<0.05 compared to the saline group (One-way ANOVA followed by Tukey's t test).

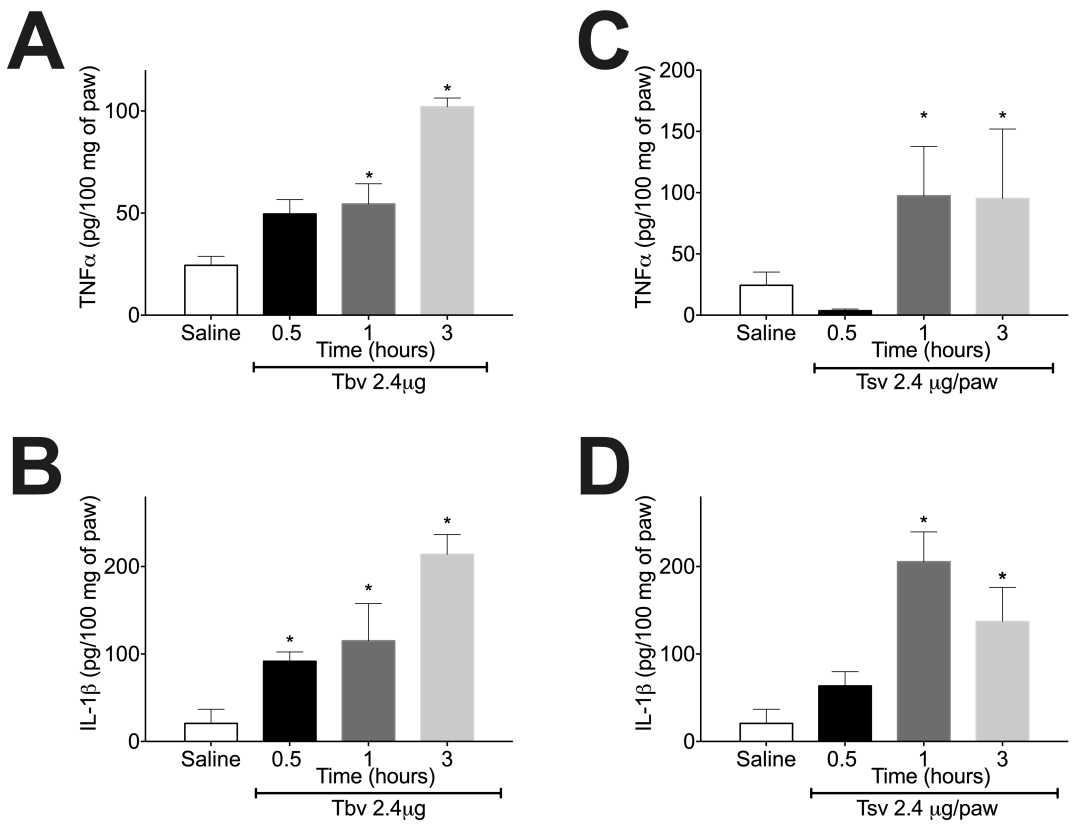

Figure 3. *Tityus bahiensis (*A and B) and *Tityus serrulatus* (C and D) venom induce TNF-a and IL-1b production in the mice paw skin. Mice received i.pl. injection of *Tityus bahiensis* (panel A and B) *or Tityus serrulatus* (panel C and D) venom (2.4 µg/paw) or saline (20 µL). Paw skin samples were collected at 0.5, 1, 3 h after stimulus with scorpion venoms, and TNF-a and IL-1b levels measured by ELISA. Results are presented as means ± s.e.m. of 6 mice per group per experiment, and are representative of 2 separated experiments. *P<0.05 compared to the saline group (One-way ANOVA followed by Tukey's t test).

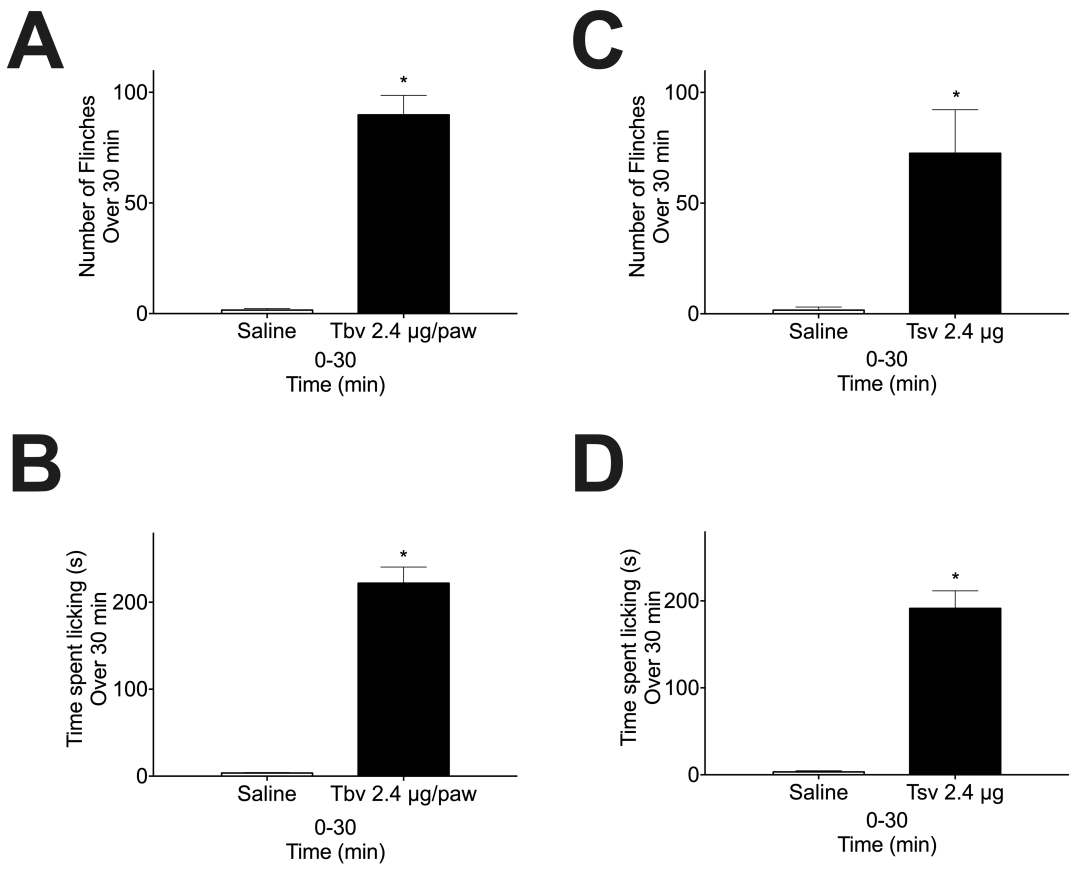

Figure 4. *Tityus bahiensis (*A and B) and *Tityus serrulatus* (C and D) venom induce overt pain-like behavior in mice. Mice received i.pl. injection of *Tityus bahiensis* (panel A and B) *or Tityus serrulatus* (panel C and D) venom (2.4 µg/paw) or saline (20 µL). The number of paw flinches and time spent licking were evaluated 0-30 min after scorpion venoms injection. Results are presented as means ± s.e.m. of 6 mice per group per experiment, and are representative of 2 separated experiments. *P<0.05 compared to the saline group (One-way ANOVA followed by Tukey's t test).

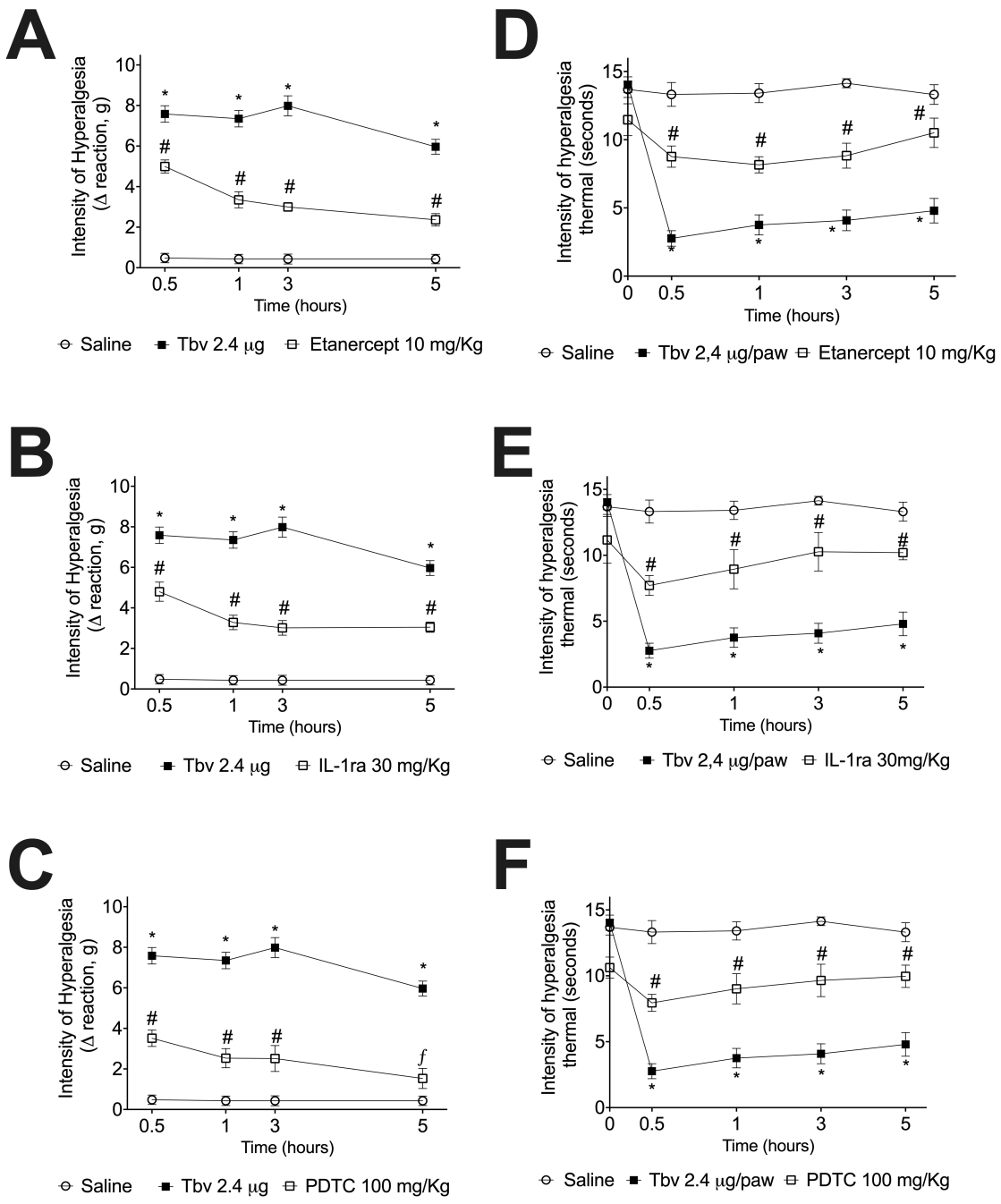

Figure 5. Systemic treatment with etanercept (A and D), L-1ra (B and E) and PDTC (C and F) reduce *Tityus bahiensis* venom-induced mechanical and thermal hyperalgesia in mice. Mice were pre-treated with etanercept (10 mg/kg, 200 µL i.p., 48 h plus 1 h, panel A and D), IL- 1ra (30 mg/kg, 200 µL i.p., 30 min, panel B and E) or PDTC (100 mg/kg, 100 µL s.c., 30 min, panel C and F) or equivalent volume of saline before i.pl. injection *Tityus bahiensis* venom (2.4

µg/paw). Mechanical and thermal hyperalgesia were evaluated after 0.5- 5 h. Results are presented as means ± s.e.m. of 6 mice per group per experiment, and are representative of 2 separated experiments. *P<0.05 compared to the saline group; #P<0.05 compared to the group saline and *Tityus bahiensis* venom group; fP<0.05 compared to *Tityus bahiensis* venom group (One-way ANOVA followed by Tukey's t test).

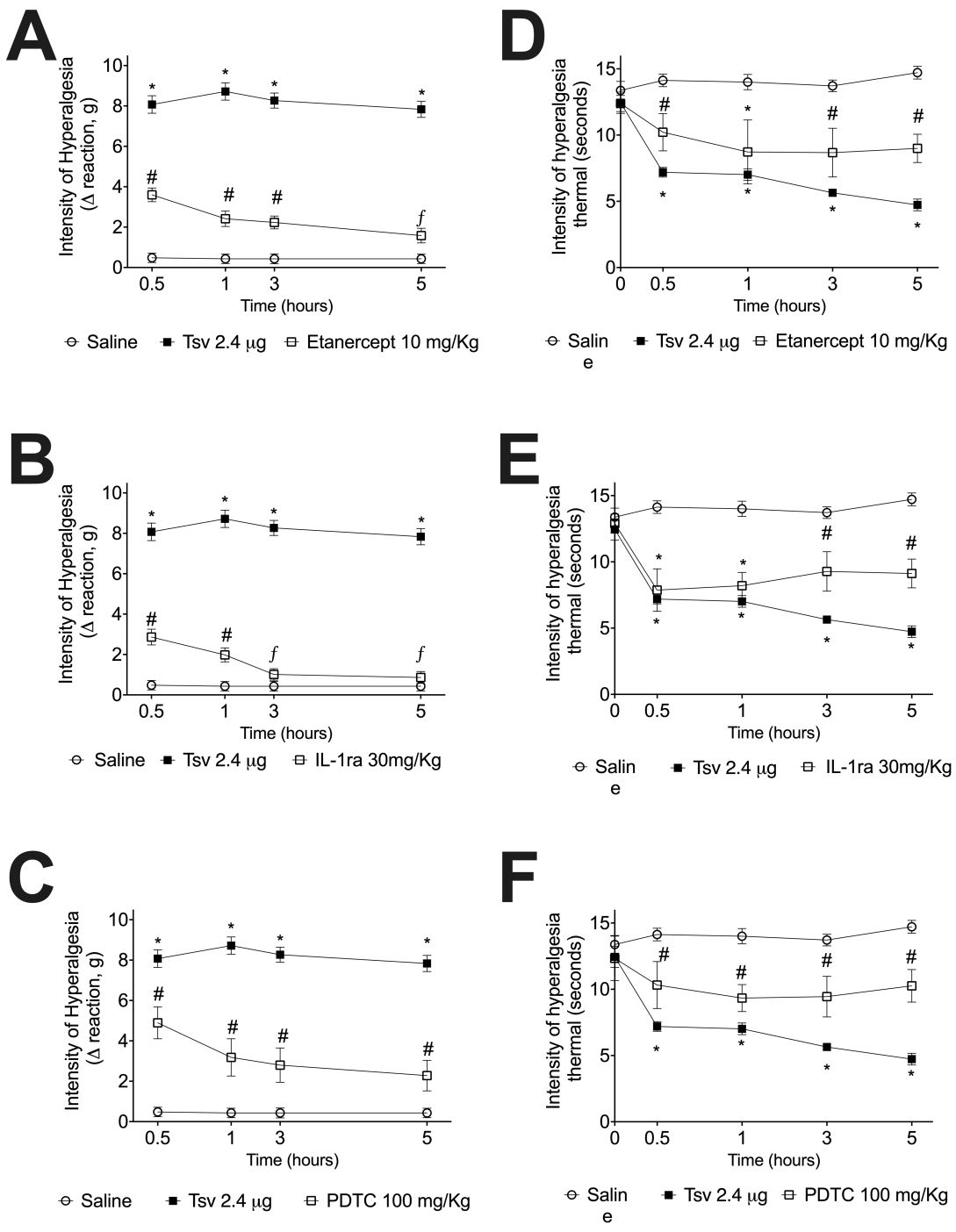

Figure 6. Systemic treatment with etanercept (A and D), L-1ra (B and E) and PDTC (C and F) reduce *Tityus serrulatus* venom-induced mechanical and thermal hyperalgesia in mice. Mice were pre-treated with etanercept (10 mg/kg, 200 μL i.p., 48 h plus 1 h, panel A and D), IL- 1ra (30 mg/kg, 200 μL i.p., 30 min, panel B and E) or PDTC (100 mg/kg, 100 μL s.c., panel C and F) or equivalent

volume of saline before i.pl. injection *Tityus serrulatus* venom (2.4 µg/paw). Mechanical and thermal hyperalgesia were evaluated after 0.5- 5 h. Results are presented as means ± s.e.m. of 6 mice per group per experiment, and are representative of 2 separated experiments. *P<0.05 compared to the saline group; #P<0.05 compared to the group saline and *Tityus serrulatus* venom group; fP<0.05 compared to *Tityus serrulatus* venom group (One-way ANOVA followed by Tukey's t test).

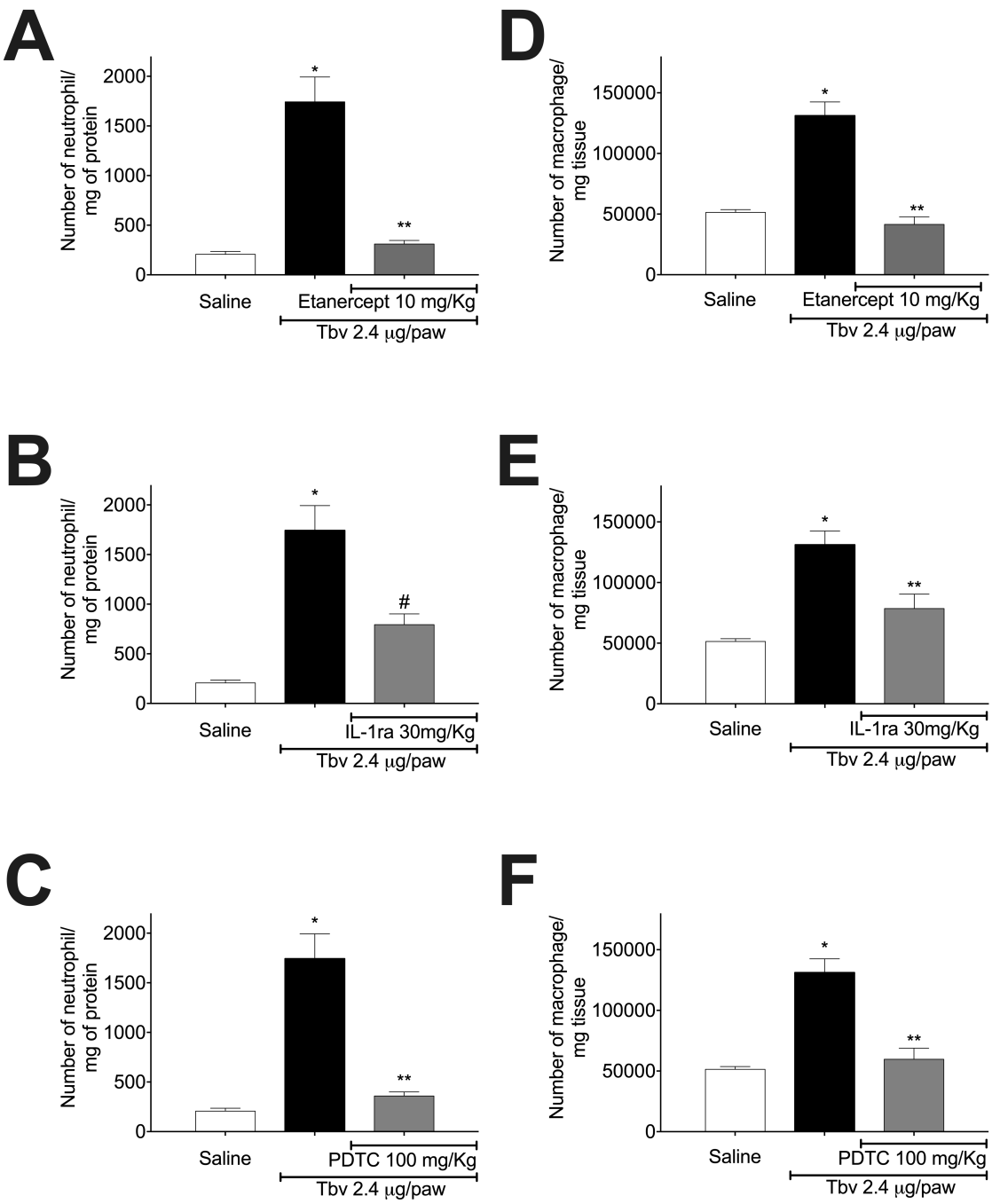

Figure 7. Pre-treatment with etanercept (A and D), L-1ra (B and E) and PDTC (C and F) reduce *Tityus bahiensis* venom-induced MPO and NAG in mice. Mice were pre-treated with etanercept (10 mg/kg, 200 µL i.p., 48 h plus 1 h, panel A and D), IL- 1ra (30 mg/kg, 200 µL i.p., 30 min, panel B and E) or PDTC (100 mg/kg, 100 µL s.c., panel C and F) or equivalent volume of saline before i.pl. injection *Tityus bahiensis* venom (2.4 µg/paw). Paw skin samples were collected

at 5h after stimulus with scorpion venoms, and MPO and NAG activity were measured. Results are presented as means ± s.e.m. of 6 mice per group per experiment, and are representative of 2 separated experiments. *P<0.05 compared to the saline group; #P<0.05 compared to the saline and *Tityus bahiensis* venom group; **P<0.05 compared to the *Tityus bahiensis* venom group (One-way ANOVA followed by Tukey's t test).

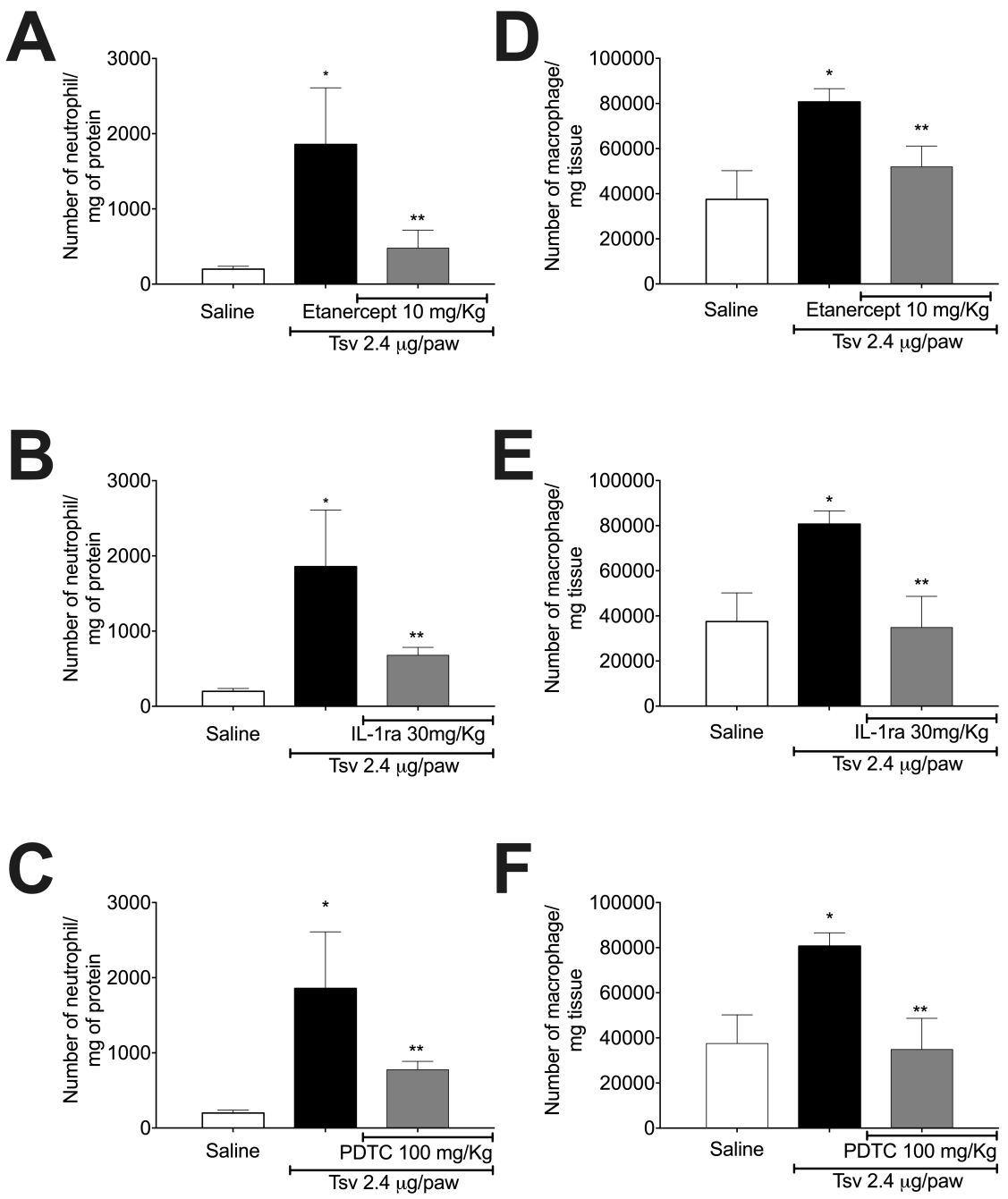

Figure 8. Pre- treatment with etanercept (A and D), L-1ra (B and E) and PDTC (C and F) reduce *Tityus serrulatus* venom-induced MPO and NAG in mice. Mice were pre-treated with etanercept (10 mg/kg, 200 µL i.p., 48 h plus 1 h, panel A and D), IL- 1ra (30 mg/kg, 200 µL i.p., 30 min, panel B and E) or PDTC (100 mg/kg, 100 µL s.c., panel C and F) or equivalent volume of saline before i.pl.

injection *Tityus serrulatus* venom (2.4 µg/paw). Paw skin samples were collected at 5h after stimulus with scorpion venoms, and MPO and NAG activity were measured. Results are presented as means ± s.e.m. of 6 mice per group per experiment, and are representative of 2 separated experiments. *P<0.05 compared to the saline group; **P<0.05 compared *Tityus serrulatus* venom group (One-way ANOVA followed by Tukey's t test).

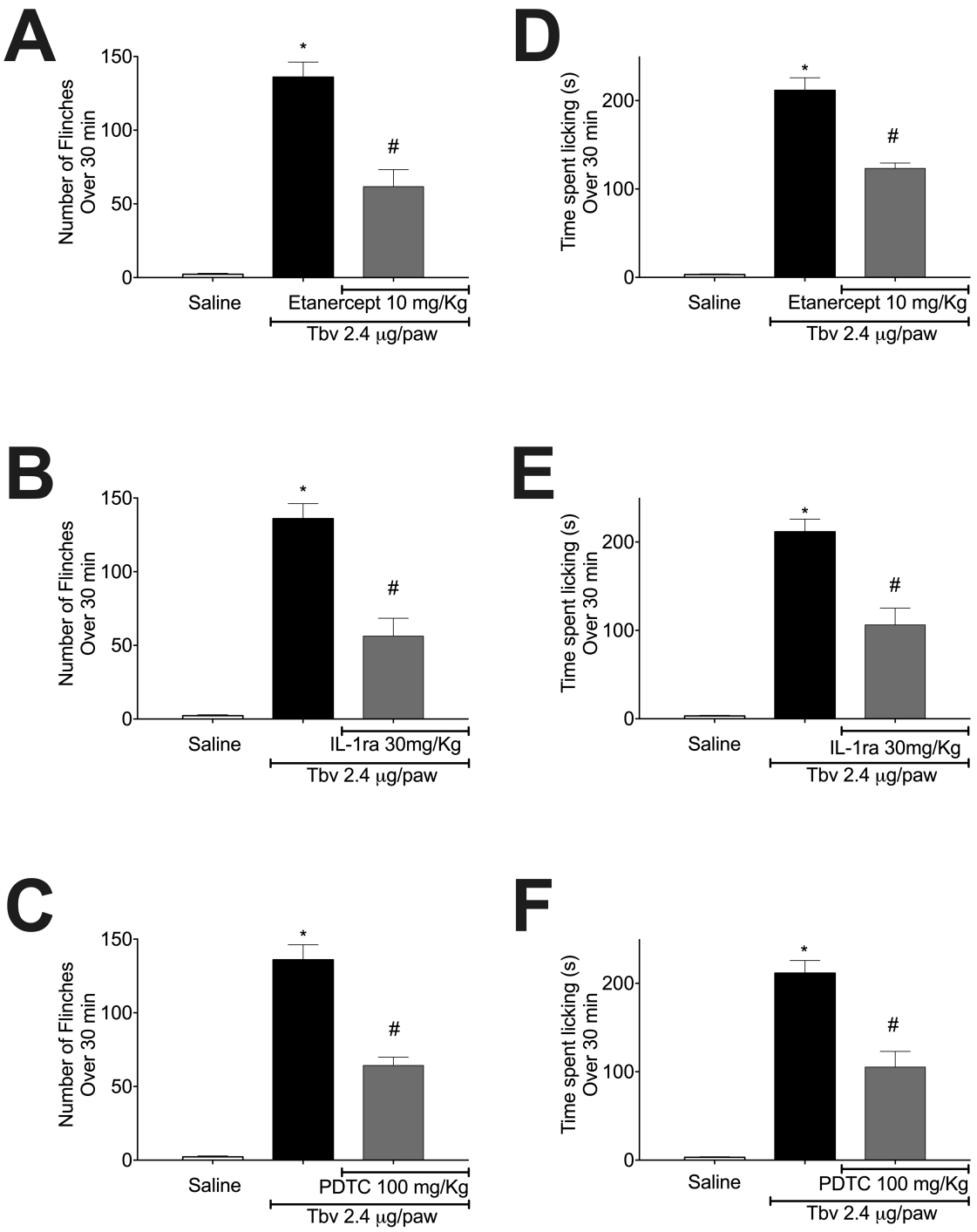

Figure 9. Pre-treatment with etanercept, L-1ra and PDTC diminish overt pain-like behaviour induced by *Tityus bahiensis* venom in mice. Mice were pre-treated with etanercept (10 mg/kg, 200 µL i.p., 48 h plus 1 h, panel A and D), IL- 1ra (30 mg/kg, 200 µL i.p., 30 min, panel B and E) or PDTC (100 mg/kg, 100 µL s.c., panel C and F) or equivalent volume of saline before i.pl. injection *Tityus*

bahiensis venom (2.4 µg/paw). The number of paw flinches and time spent licking were evaluated 0-30 min after scorpion venoms injection. Results are presented as means ± s.e.m. of 6 mice per group per experiment, and are representative of 2 separated experiments. *P<0.05 compared to the saline group; #P<0.05 compared to the saline and *Tityus bahiensis* venom group (One-way ANOVA followed by Tukey's t test).

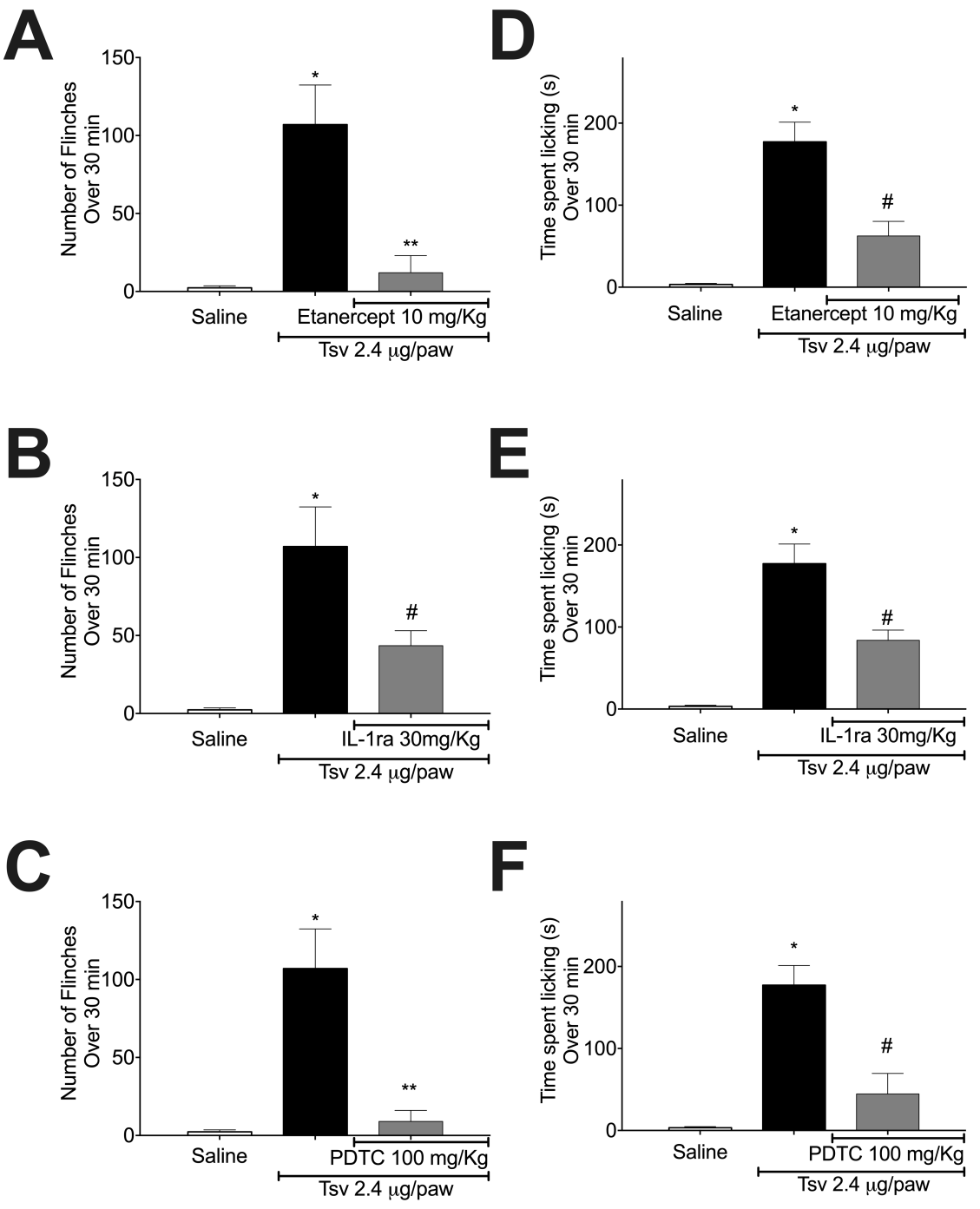

Figure 10. Pre-treatment with etanercept, L-1ra and PDTC diminish overt pain-like behaviour induced by *Tityus serrulatus* venom in mice. Mice were pre-treated with etanercept (10 mg/kg, 200 µL i.p., 48 h plus 1 h, panel A and D), IL- 1ra (30 mg/kg, 200 µL i.p., 30 min, panel B and E) or PDTC (100 mg/kg, 100 µL s.c., panel C and F) or equivalent volume of saline before i.pl. injection *Tityus*

serrulatus venom (2.4 µg/paw). The number of paw flinches and time spent licking were evaluated 0-30 min after scorpion venoms injection. Results are presented as means ± s.e.m. of 6 mice per group per experiment, and are representative of 2 separated experiments. *P<0.05 compared to the saline group; #P<0.05 compared to the saline and *Tityus serrulatus* venom group; **P<0.05 compared to *Tityus serrulatus* venom group (One-way ANOVA followed by Tukey's t test).

5 CONCLUSÃO

No primeiro artigo, **"Spinal cord microglia and astrocyte mediate jararhagin-induced mechanical hyperalgesia in mice"** demonstramos a participação das células da glia na hiperalgesia mecânica induzida pela jararagina e o mecanismo envolve a produção de citocinas pró-inflamatórias na medula espinal via NFkB. No segundo artigo, **"Jararhagin-induced mechanical hyperalgesia depends on spinal activation of MAP kinases in mice"** demonstramos que a ativação das MAPKs é um mecanismo importante no desenvolvimento dor induzida por essa metaloproteinase. Esses dados demonstram pela primeira vez a importância das células da glia e das MAPks na dor induzida pela jararagina e, ao mesmo tempo, contribuem para um melhor entendimento sobre os mecanismos espinais envolvidos na dor induzida por venenos de serpentes. Entretanto, ainda são necessários mais estudos para demonstrar o papel de cada célula nesse processo e, bem como, em qual tipo celular as MAKPs são ativadas.

No terceiro artigo, **"Peripheral mechanisms involved in the nociception triggered by *Tityus bahiensis* and *Tityus serrulatus* venom"** demonstramos o recrutamento de neutrófilos e macrófagos, a ativação do NFkB e a produção de TNF-α e IL-1β desempenham um papel importante na nocicepção induzida pelos venenos de *Tityus bahiensis* e *Tityus serrulatus.* Dessa maneira, essas descobertas podem contribuir para o desenvolvimento de melhores terapias para o escorpionismo.

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APÊNDICE A- Multifunctional Toxins in Snake Venoms and Therapeutic Implications: From Pain to Hemorrhage and Necrosis

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REVIEW
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Multifunctional Toxins in Snake Venoms and Therapeutic Implications: From Pain to Hemorrhage and Necrosis

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Animal venoms have evolved over millions of years for prey capture and defense from predators and rivals. Snake venoms, in particular, have evolved a wide diversity of peptides and proteins that induce harmful inflammatory and neurotoxic effects including severe pain and paralysis, hemotoxic effects, such as hemorrhage and coagulopathy, and cytotoxic/myotoxic effects, such as inflammation and necrosis. If untreated, many envenomings result in death or severe morbidity in humans and, despite advances in management, snakebite remains a major public health problem, particularly in developing countries. Consequently, the World Health Organization recently recognized snakebite as a neglected tropical disease that affects ∼2.7 million p.a. The major protein classes found in snake venoms are phospholipases, metalloproteases, serine proteases, and three-finger peptides. The mechanisms of action and pharmacological properties of many snake venom toxins have been elucidated, revealing a complex multifunctional cocktail that can act synergistically to rapidly immobilize prey and deter predators. However, despite these advances many snake toxins remain to be structurally and pharmacologically characterized. In this review, the multifunctional features of the peptides and proteins found in snake venoms, as well as their evolutionary histories, are discussed with the view to identifying novel modes of action and improving snakebite treatments.

Keywords: snake venoms, multifunctional toxins, pathological mechanisms, evolution, snakebite treatment, pain, hemotoxicity, myotoxicity

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INTRODUCTION

The composition and evolutionary histories of animal venoms have fascinated the scientific community for centuries. Venoms have evolved over millions of years to facilitate prey capture and/or defense from predators and rivals. Snake venoms, in particular, likely originated in the Cenozoic Era (Fry, 2005; Fry et al., 2006), and they are amongst the most well-characterized of animal venoms, comprising a complex mixture of toxic, and pharmacologically-active proteins and