KASSYO LENNO SOUSA DANTAS

MARESINA 2 REDUZ DOR E INFLAMAÇÃO INDUZIDAS PELA PEÇONHA DE *Bothrops jararaca* EM CAMUNDONGOS.

Londrina - PR
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DANTAS, Kassyo Lenno Sousa. **Maresina 2 reduz dor e inflamação induzidas pela peçonha de *Bothrops jararaca* em camundongos**. 2023. 56 f. Dissertação (Mestrado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2023.

RESUMO

As picadas de serpentes podem induzir inflamação e dor. A peçonha de *Bothrops jararaca* (BjV) causa dor intensa e prolongada que não é revertida pelo antiveneno, além de hemorragia, infiltrado de células inflamatórias e edema. A Maresina 2 (MaR2) é um Mediador Lipídico Pro-Resolução Especializado (SPM's) com propriedades anti-inflamatórias, pró-resolutivas e analgésicas. Neste estudo, investigamos a eficácia da MaR2 na redução da dor e inflamação induzidas por BjV em camundongos. Para isso, foi utilizado a avaliação da hiperalgesia mecânica (versão eletrônica dos filamentos de von Frey), a hiperalgesia térmica (teste de placa quente) e a distribuição de peso (suporte de peso estático), bem como a atividade da mieloperoxidase (ensaio MPO), a produção de ânion superóxido (O2-) (ensaio NBT), níveis totais de antioxidantes (ensaio ABTS) e os níveis de citocinas TNF-α, IL-1β e IL-6 no tecido plantar de camundongos após injeção intraplantar de BjV em diferentes doses (0.01, 0.1 e 1 µg). O pré-tratamento intraperitoneal com MaR2 (0.3, 1 e 3 ng) reduziu de maneira dose-dependente a hiperalgesia mecânica e térmica, normalizou a distribuição do peso, inibiu a atividade da MPO, a produção de O2- e promoveu o aumento dos níveis totais de antioxidantes no tecido, bem como a redução dos níveis de TNF-α, IL-1β e IL-6. Em modelo de peritonite induzida pela injeção intraperitoneal de BjV em diferentes doses (1, 3 e 5 µg), avaliamos o recrutamento de leucócitos (contagem de leucócitos totais), a hemorragia (contagem total de eritrócitos), os níveis de espécies reativas de oxigênio (ROS) e óxido nítrico intracelular (NO) (ensaio DCF e DAF, respectivamente), produção de O2- (ensaio NBT e células NBT-positivas) e a expressão de mRNA de gp91phox e óxido nítrico sintase induzível (iNOS) (RT-qPCR). O pré-tratamento intraperitoneal com MaR2 (0.1, 1 e 10 ng) reduziu de maneira dose-dependente o recrutamento de leucócitos, a hemorragia, os níveis de ROS, NO e O2-, bem como a expressão de mRNA de gp91phox e iNOS. Portanto, usando modelos de dor inflamatória na pata e a peritonite, demonstramos que a MaR2 é um mediador promissor para o tratamento da dor e inflamação induzidas por BjV.

Palavras-chave: Maresina 2; Peçonha de *Bothrops jararaca*; Dor; Inflamação.

DANTAS, Kassyo Lenno Sousa. **Maresin 2 reduces pain and inflammation induced by *Bothrops jararaca* venom in mice.** 2023. 56 p. Dissertation (Master's in Experimental Pathology) - State University of Londrina, Londrina, 2023.

ABSTRACT

Snakebites can induce inflammation and pain. The venom of *Bothrops jararaca* (BjV) causes intense and prolonged pain that is not reversed by the antivenon, as well as hemorrhage, inflammatory cell infiltration, and edema. Maresin 2 (MaR2) is a Specialized Pro-Resolving Mediator (SPM) with anti-inflammatory, pro-resolving, and analgesic properties. In this study, we investigated the efficacy of MaR2 in reducing pain and inflammation induced by BjV in mice. Mechanical hyperalgesia assessment (electronic von Frey filaments), thermal hyperalgesia (hot plate test), and weight distribution (static weight-bearing) were used, as well as myeloperoxidase activity (MPO assay), superoxide anion (O2-) production (NBT assay), total antioxidant levels (ABTS assay), and the levels of cytokines TNF-α, IL-1β, and IL-6 in mouse paw tissue after intraplantar injection of BjV at different doses (0.01, 0.1, and 1 μg). Intraperitoneal pre-treatment with MaR2 (0.3, 1, and 3 ng) dose-dependently reduced mechanical and thermal hyperalgesia, normalized weight distribution, inhibited MPO activity, reduced O2- production, and increased total antioxidant levels in the tissue, as well as reducing TNF-α, IL-1β, and IL-6 levels. In a peritonitis model induced by intraperitoneal injection of BjV at different doses (1, 3, and 5 μg), we evaluated leukocyte recruitment (total leukocyte count), hemorrhage (total erythrocyte count), levels of reactive oxygen species (ROS), and intracellular nitric oxide (NO) (DCF and DAF assays, respectively), O2- production (NBT assay and NBT-positive cells), and the mRNA expression of gp91phox and inducible nitric oxide synthase (iNOS) (RT-qPCR). Intraperitoneal pre-treatment with MaR2 (0.1, 1, and 10 ng) dose-dependently reduced leukocyte recruitment, hemorrhage, ROS, and NO levels, as well as the mRNA expression of gp91phox and iNOS. Therefore, using models of paw inflammatory pain and peritonitis, we demonstrated that MaR2 is a promising mediator for the treatment of pain and inflammation induced by BjV.

Keywords: Maresin 2; *Bothrops jararaca* venom; Pain; Inflammation.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

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12-LOX	12-lipoxigenase humana
ATP	Adenosina Trifosfato
BPP	Peptídeo Potenciador de Bradicinina
CaNa2EDTA	Cálcio - Sódio - Di-etilenotriamina-pentaacetato
CLRs	Receptores Tipo C-type Lectin
CRISP	Proteínas Secretoras Ricas em Cisteína
CTL	Lectinas do Tipo C/snaclecs
CXCL1	C-X-C Ligante 1
CXCL2	C-X-C Ligante 2
DAMPs	Padrões Moleculares Associados a Danos
DHA	Ácido Docosahexaenóico
DIS	Desintegrinas
DNA	Ácido Desoxirribonucleico
DPA	Ácido Docosapentaenóico
EP1-4	Receptores PGE2 1–4
EPA	Ácido Eicosapentaenóico
FASL	Ligante de Fas
FoxO3a	Fator de Transcrição da Família FoxO
Fpr1	Receptor de Peptídeo Formil 1
HMGB1	High Mobility Group Box 1
IASP	Associação Internacional para o Estudo da Dor
ICAM-1	Molécula de Adesão Intercelular-1
ICOS	Ligante do Coestimulador Induzível
IL-1R1	Receptor 1 de IL-1

IL-1β	Interleucina-1 beta
IL-5	Interleucina 5
IL-6	Interleucina 6
LAAO	Oxidases de Aminoácidos
LFA-1	Antígeno Associado à Função de Linfócitos 1
LPS	Lipopolissacarídeos
LRRC33	Leucine Rich Repeat Containing 33
LTB4	Leucotrieno B4
MaR1	Maresina 1: 7R,14S-dihydroxy-docosa-4Z,8E,10E,12Z,16Z,19Z-Hexaenoic Acid
MaR2	Maresina 2: 13R,14S-dihydroxy-4Z,7Z,9E,11E,16Z,19Z-Hexaenoic Acid
NF-κB	Fator Nuclear kappa- B
NGF	Fator de Crescimento de Nervos
NLRs	Receptores Tipo NOD-like
PAMPs	Padrões Moleculares Associados a Patógenos
PCNA	Fatores Associados ao Antígeno Nuclear Proliferativo
PDL1	Ligante de Morte Programada 1
PGE2	Prostaglandina E2
PLA2	Fosfolipases A2
PRRs	Receptores de Reconhecimento de Padrões
PUFAS	Ácidos Graxos Poliinsaturados
RLRs	Receptores Tipo RIG-I-like
RNA	Ácido Ribonucleico
SAB	Soro Antibotrópico
SPMs	Mediadores Lipídicos Pro-Resolução Especializados
SRs	Receptores Tipo Scavenger
SVMP	Metaloproteinases da Peçonha de Serpentes

SVSP	Serinoproteases da Peçonha de Serpentes
TGF-β	Fator Transformador de Crescimento Beta
TLRs	Receptores do Tipo Toll-like
TNFR1	Receptor 1 do TNFα
TNF-α	Fator de Necrose Tumoral alfa
TRP	Receptores de Potencial Transitório
TRPA1	Receptor Potencial Transitório de Anquirina 1
TRPV1	Receptor Potencial Transitório de Vanilóide 1
VCAM-1	Molécula de Adesão de Células Vasculares-1
VEGF	Fator de Crescimento Endotelial Vascular
VLA-4	Integrina α4β1

SUMÁRIO

1. INTRODUÇÃO GERAL

Os organismos vivos enfrentam continuamente desafios decorrentes da exposição a fatores ambientais que representam riscos físicos, químicos e biológicos. Para contornar esses desafios, em uma escala evolutiva, tais organismos desenvolveram mecanismos que os permitem se adaptar ao ambiente (Pancer e Cooper, 2006).

Animais vertebrados, objetos de estudo deste trabalho, desenvolveram um sofisticado sistema de defesa, que tem como objetivo auxiliar o indivíduo a se defender de estímulos potencialmente nocivos. O sistema de defesa, também chamado de sistema imune, baseia-se em duas funções essenciais: 1) a identificação e diferenciação de componentes próprios de componentes não próprios (isto é, havendo autotolerância); 2) uma elaborada ativação celular e de vias associadas a síntese de moléculas, que de forma direta ou indireta eliminam o agente ou fenômeno agressor (Turvey e Broide, 2010).

De forma geral, o sistema imune é dividido em dois grupos distintos, mas, apesar disso, comunicam-se intimamente (Abbas, Pillai e Lichtman, 2019). A imunidade inata, considerada a primeira linha de defesa do organismo, é dita como a mais universal, de ação rápida e eficaz (Kaur e Secord, 2021). Composta essencialmente por barreiras epiteliais, proteínas do sistema complemento, células fagocíticas como neutrófilos, monócitos e macrófagos, células essencialmente liberadoras de mediadores inflamatórios, como mastócitos, basófilos e eosinófilos e por células natural killer (Abbas, Pillai e Lichtman, 2019).

A imunidade adaptativa, é conhecida por ser uma resposta tardia, visto que opera reconhecendo detalhes sutis de microrganismos, sendo mais específica comparada a imunidade inata, composta principalmente por linfócitos T e linfócitos B (Abbas, Pillai e Lichtman, 2019). Neste contexto, a complexa atividade do sistema imune desempenha um papel fundamental na resposta inflamatória do organismo, um processo intrinsecamente ligado à manutenção da homeostase (Kaur e Secord, 2021).

1.1 Processo inflamatório

A importância do sistema imune e de sua complexa atividade é amplamente reconhecida desde meados do século XX, com a interação entre citocinas, quimiocinas, mediadores lipídicos e células atuando de forma coordenada para manter o equilíbrio da homeostase (Rocha e Silva, 1994). A complexa resposta do sistema imune a agentes e eventos nocivos são acompanhadas, muitas vezes, por um processo que apresenta sinais característicos, conhecidos como os sinais cardinais da inflamação. Descritos pela primeira vez no primeiro século D.C por Aulus Cornelius Celsus, os sinais cardinais se manifestam como calor, rubor/vermelhidão, edema e dor e em 1858, Rudolph Virchow acrescentou um quinto sinal, o distúrbio/perca da função (Rocha e Silva, 1994).

Em resposta a uma agressão, as células do sistema imune inato respondem rapidamente por meio de um mecanismo de reconhecimento de padrões capaz de identificar rapidamente ameaças e iniciar uma resposta imune apropriada (Pradeu e Cooper, 2012). A inflamação é uma parte principal da resposta imune inata, operando para assegurar a homeostase e promover a restauração dos tecidos danificados (Medzhitov, 2008). Há diferentes fases da inflamação, cada uma com características e mecanismos distintos. A inflamação aguda é definida como uma resposta rápida e transitória a uma lesão tecidual ou infecção. Esse processo envolve a ativação celular, principalmente por meio de Receptores de Reconhecimento de Padrões (PRRs), presentes em fagócitos, como macrófagos residentes e neutrófilos, conhecidas por geralmente serem as primeiras a chegarem ao local de uma lesão ou infecção (Kolaczkowska e Kubes, 2013).

Estruturas moleculares conservadas encontradas em patógenos, como componentes da parede celular bacteriana, ácidos nucleicos virais ou lipopolissacarídeos (LPS), são conhecidas como Padrões Moleculares Associados a Patógenos (PAMPs) (Zindel e Kubes, 2020a). Além disso, algumas moléculas podem ser liberadas por células danificadas ou mortas durante processos de estresse celular e metabólico. Um exemplo disso são as alarminas, como o ATP extracelular, outros metabólitos como o ácido úrico, a “High Mobility Group Box 1” (HMGB1), as proteínas de choque térmico (como HSP60 e HSP70), o ácido desoxirribonucleico

(DNA) e o ácido ribonucleico (RNA). Essas moléculas são conhecidas como Padrões Moleculares Associados a Danos (DAMPs) (Zindel e Kubes, 2020a).

Tanto PAMPs como DAMPs são identificados pelos PRRs, como receptores do tipo Toll-like (TLRs), receptores tipo NOD-like (NLRs), receptores tipo RIG-I-like (RLRs), receptores tipo C-type lectin (CLRs) e receptores tipo scavenger (SRs) que são expressos na grande maioria das células, principalmente em fagócitos (Abbas, Pillai e Lichtman, 2019).

De maneira clássica, a inflamação aguda é iniciada com a ativação de PRRs, principalmente de células imunes residentes, como macrófagos, mastócitos e células dendríticas (Abbas, Pillai e Lichtman, 2019). A detecção de PAMPs e DAMPs culmina na ativação desses receptores, desencadeando uma série de sinais intracelulares que levam, por exemplo, na ativação e translocação do Fator Nuclear kappa-B (NF-κB) para o núcleo, promovendo a expressão de uma variedade de genes, e posterior produção e liberação de citocinas e quimiocinas pró-inflamatórias (como Fator de Necrose Tumoral alfa [TNF-α], Interleucina [IL] - 1 beta [IL-1β], IL-6, mediadores lipídicos pró-inflamatórios (Leucotrieno B4 [LTB4] e Prostaglandina E2 [PGE2]), amplificando a resposta imune (Abbas, Pillai e Lichtman, 2019).

Além disso, o ambiente pró-inflamatório acarreta uma série de eventos nos vasos sanguíneos adjacentes, como o aumento do fluxo sanguíneo e aumento da permeabilidade endotelial, mediados essencialmente por aminas vasoativas como histamina e serotonina (Majno, Palade e Schoefl, 1961). A ativação do endotélio contribui para expressão de moléculas de adesão (Molécula de Adesão Intercelular-1 [ICAM-1], Molécula de Adesão de Células Vasculares-1 [VCAM-1]), selectinas (P-seletina e E-selectina), ligantes para integrinas e quimiocinas (como C-X-C ligante 1 [CXCL1] e C-X-C ligante 2 [CXCL2]) no lúmen do vaso sanguíneo. Isso promove a interação com moléculas de adesão expressas na superfície dos leucócitos circulantes (como Antígeno Associado à Função de Linfócitos 1 [LFA-1] e Antígeno Muito Tardio 4 [VLA-4]), promovendo a atração e direcionamento de leucócitos para o sitio de inflamação através de um gradiente espacial de quimioatraentes (Abbas, Pillai e Lichtman, 2019; Petrie Aronin et al., 2017).

Os neutrófilos, por exemplo, são recrutados rapidamente para o tecido inflamado (Margraf, Ley e Zarbock, 2019). O ambiente inflamatório local induz a

expressão do FoxO3a, que, por sua vez, prolonga a vida útil dos neutrófilos, inibindo a ativação do promotor do FasL (Ligante de Fas) nos neutrófilos. Esse promotor está envolvido na promoção da apoptose em resposta, por exemplo, ao TNF-α e à IL-1. Além disso, o Fator de Transcrição da Família FoxO (FoxO3a) estimula a ativação do NF-κB, bem como a expressão de citocinas pró-inflamatórias e a produção de espécies reativas de oxigênio (Jonsson, Allen e Peng, 2005).

A inflamação aguda desempenha um papel fundamental na resposta do organismo a uma variedade de agressões, incluindo a picada de serpentes peçonhentas, como a *Bothrops jararaca*. Essas serpentes são conhecidas por causar uma reação inflamatória significativa no local da picada, muitas vezes resultando em rubor, edema e dor intensa (Ferraz et al., 2021).

1.2 *Bothrops jararaca*

Desde os tempos antigos, os venenos foram usados em várias culturas para diversos fins, como armas de guerra, ferramentas de caça, ingredientes em rituais e até mesmo na medicina (Casewell et al., 2013). A grande maioria das peçonhas de animais é constituída por coquetéis extremamente complexos de compostos bioativos. Geralmente, uma mistura diversificada de proteínas e peptídeos, frequentemente chamados de toxinas, além de sais e componentes orgânicos como aminoácidos e neurotransmissores (Mohamed ABD, Garcia S e Stockand, 2019).

Serpentes do gênero *Bothrops* são encontradas em diferentes regiões da América Central e do Sul. A distribuição geográfica abrange uma ampla área que se estende desde o sul do México, passando pela América Central até a América do Sul (Sant'Ana Malaque e Gutiérrez, 2015). Diferentes espécies de *Bothrops* podem ser encontradas em diferentes habitats, incluindo florestas tropicais, cerrados, savanas e áreas de transição entre esses ecossistemas. No Brasil, por exemplo, espécies como a *Bothrops jararaca* são encontradas em várias regiões do país (Sant'Ana Malaque e Gutiérrez, 2015).

A *Bothrops jararaca*, também chamada de Jararaca ou Jararaca-da-mata, é uma serpente peçonhenta que pertence à família Viperidae do gênero *Bothrops* e é

uma das espécies de serpentes mais conhecidas e estudadas no país (Costa e Bérnils, 2018). Possui características como cabeça triangular, corpo robusto e cauda curta e geralmente apresenta coloração variando entre tons de marrom, cinza e verde. Possui hábitos noturnos e no período diurno costuma se abrigar em locais como tocas, troncos ocos, folhagens densas e vegetação rasteira para evitar a exposição ao sol e a predadores. Por ser carnívora, se alimenta principalmente de pequenos vertebrados, como roedores, aves, lagartos e anfíbios (Gomes, N. & G. Puorto. 1993).

Acidentes ofídicos envolvendo serpentes representam um sério problema de saúde pública em diversos países tropicais e subtropicais (Kasturiratne et al., 2008). Devido ao impacto significativo em termos sociais e econômicos causado por esses acidentes, em junho de 2017, durante a 10ª reunião do Grupo Consultivo Estratégico e Técnico sobre Doenças Tropicais Negligenciadas, realizada em Genebra, os acidentes ofídicos foram incluídos como doenças tropicais negligenciadas prioritárias pela OMS (Chippaux, 2017). Dentre os países mais afetados, o Brasil se destaca com o maior número de casos na América do Sul (Chippaux, 2015), com cerca de 69,3% associados a serpente *Bothrops jararaca* (Instituto Butantan, 2022).

A patogênese do envenenamento botrópico é complexa em razão dos diversos peptídeos e proteínas com propriedades farmacologicamente ativas que desencadeiam uma ampla gama de sinais e sintomas nas vítimas (Tasoulis e Isbister, 2017). De maneira geral, a avaliação da composição proteômica relativa da peçonha de *Bothrops* revela uma preponderância de fosfolipases A2 (PLA2), metaloproteinases da peçonha de serpentes (SVMP), serinoproteases da peçonha de serpentes (SVSP) e, em segundo plano, famílias de proteínas como proteínas secretoras ricas em cisteína (CRiSP), oxidases de aminoácidos l (LAAO), lectinas do tipo C/snaclecs (CTL), desintegrinas (DIS), Peptídeo Potenciador de Bradicinina (BPP) e Fator de Crescimento Endotelial Vascular (VEGF) (Albuquerque et al., 2020; Tasoulis e Isbister, 2017).

Manifestações locais são associadas principalmente a ação de PLA2, SVMP e SVSP (Gutiérrez e Lomonte, 1995). A picada pode causar dor local intensa e prolongada (Ferraz et al., 2021; Kondo et al., 2022), que não é revertida pelo antiveneno (Picolo et al., 2002), mionecrose (Gay et al., 2005), hemorragia (Gutiérrez et al., 1998; Nadur-Andrade et al., 2012), infiltrado de células inflamatórias

(Almeida et al., 2020; Cedro et al., 2018) e edema (Nadur-Andrade et al., 2012) também são observados após a picada.

O tratamento específico e preconizado para casos de envenenamento por serpentes do gênero *Bothrops* é o uso do soro antibotrópico (SAB). A eficácia de SAB é avaliada por meio do estudo da sua capacidade de neutralizar a atividade letal da peçonha de *Bothrops jararaca*, considerada como a peçonha de referência para esse propósito (Muniz et al., 2000). A administração intravenosa de SAB neutraliza as ações sistêmicas, mas possui baixa eficácia na reversão dos sintomas locais (Battellino et al., 2003). De modo geral, além do uso do SAB, o uso de medicamentos anti-inflamatórios, analgésicos e antibioticoterapia (como diclofenaco, paracetamol e cloranfenicol, respectivamente) são comumente empregados, no entanto, muitas vezes esses medicamentos apresentam toxicidade e reações adversas indesejadas (Ministério da Saúde. Fundação Nacional de Saúde., 2001).

Abordagens farmacológicas adicionais têm sido investigadas para aprimorar o gerenciamento das reações locais associadas aos envenenamentos. Isso inclui o uso de substâncias como o Cálcio (Ca) - Sódio (Na) - Di-etilenotriamina-pentaacetato (EDTA) (CaNa2EDTA), que atua como um agente quelante, inibindo os sítios ativos de metaloproteinases hemorrágicas, bem como inibidores sintéticos de metaloproteinases da matriz e heparina. Essas terapias complementares visam melhorar o tratamento de complicações locais decorrentes de envenenamentos (Battellino et al., 2003).

1.3 Dor inflamatória

Dos sinais cardinais da inflamação, talvez a dor seja aquele que mais rapidamente se manifesta e impacta o organismo nos acidentes ofídicos envolvendo a *Bothrops jararaca*. Em 1965, Melzack-Wall apresentou a teoria do controle de comportamento da dor, conhecida como Teoria do Controle de Portão (Melzack e Wall, 1965), revolucionando o entendimento da dor e fornecendo uma base para pesquisas posteriores. Atualmente, a Associação Internacional para o Estudo da Dor – IASP, compreende a dor como “uma experiência sensorial e emocional

desagradável associada ou semelhante à associada a um dano tecidual real ou potencial.” (Raja et al., 2020).

De modo geral, a dor é complexa e envolve tanto aspectos sensoriais como cognitivos e emocionais (Loeser e Melzack, 1999). Após uma lesão nos tecidos e o desenvolvimento de um estado inflamatório, os nociceptores (receptores sensoriais responsáveis pela detecção da dor) e neurônios sensoriais podem se tornar sensibilizados, resultando em uma resposta dolorosa a estímulos que antes eram leves ou inofensivos. Essa condição é comumente conhecida como hiperalgesia, que se refere a uma resposta exacerbada a estímulos normalmente dolorosos, ou alodinia, que é a percepção de dor em resposta a estímulos que normalmente não seriam dolorosos (Verri et al., 2006).

Neurônios sensoriais e nociceptores expressam variados receptores e canais iônicos, onde vias de sinalização intracelular específicas promovem a fosforilação e/ou ativação de canais iônicos (como canais de sódio v 1.7, Na v 1.8, Na v1.9) e Receptores de Potencial Transitório (TRP) (TRPV1 e TRPA1), levando ao aumento da geração de potencial de ação e sensibilidade à dor. Mastócitos liberam fator de crescimento de nervos (NGF), IL-5, histamina e serotonina (5-HT), que atuam através do receptor TrkA, receptor IL-5R, receptor de histamina 2 (H2) e receptores 5-HT2, respectivamente, os quais também tem papel importante na sensibilização e ativação das terminações dos neurônios aferentes primários (Cunha et al., 2005; Ferreira et al., 1988; Levine et al., 1984; Vivancos, Parada e Ferreira, 2003).

Além disso, mastócitos, macrófagos e neutrófilos liberam TNFα, IL-1β e IL-6. TNFα atua sobre o receptor 1 do TNFα (TNFR1) levando à fosforilação dos canais de sódio NaV 1.9, enquanto o receptor 1 de IL-1 (IL-1R1) aumenta a expressão do receptor TRPV1 nos nociceptores. A IL-6 liga-se ao receptor gp130 nos nociceptores, elevando a expressão de TRPV1 e TRPA1, o que amplifica a capacidade de resposta a estímulos térmicos e produtos químicos reativos. Ainda, a PGE2 liberada por macrófagos e outras células do sistema imunológico age através dos receptores PGE2 1–4 (EP1-4) nos neurônios nociceptores, contribuindo para a sensibilização à dor (Pinho-Ribeiro, Verri e Chiu, 2017).

Diferentes moléculas derivadas de patógenos ou animais peçonhentos através de mecanismos distintos podem estimular a sensibilidade à dor. Por exemplo, os peptídeos N-formilados e a alfa-hemolisina de *Staphylococcus aureus* induzem respostas neuronais diretas através de mecanismos distintos: peptídeos formil através do Receptor de Peptídeo Formil 1 (Fpr1) e alfa-hemolisina através da montagem de poros levando ao influxo iônico (Chiu et al., 2013).

O LPS induz hiperalgesia mecânica dependente da ativação do receptor TLR4 e do recrutamento da molécula adaptadora MyD88 (Calil et al., 2014). A capsaicina estimula seletivamente os neurônios nociceptivos e é amplamente usada para estudar eventos relacionados à dor (Fattori et al., 2016). A peçonha da serpente *Bothrops jararaca* causa dor local intensa e prolongada (Ferraz et al., 2021; Kondo et al., 2022), através de vias que incluem a ativação do NFκB e subsequente produção das citocinas pró-hiperalgésicas TNF-α e IL-1β (Ferraz et al., 2021).

As reações a peçonha exemplificam a complexidade do mecanismo fisiológico da dor. De modo geral, o mecanismo envolve alguns passos gerais: transdução, condução, transmissão e percepção dos estímulos nóxicos (Basbaum et al., 2009; Garland, 2012). O processo inicia-se com a conversão de um estímulo na periferia, pelas fibras sensoriais nociceptivas, incluindo fibras aferentes primárias não mielinizadas (fibras C) e mielinizadas (fibras Aδ). Essas fibras estão conectadas a neurônios pseudounipolares de primeira ordem, cujos corpos celulares estão localizados nos gânglios da raiz dorsal. A partir daí, ocorre a geração de um potencial de ação que é transmitido em direção aos cornos da raiz dorsal da medula espinal ou ao núcleo trigeminal do tronco encefálico. Em seguida, os neurotransmissores excitatórios desempenham um papel crucial na transmissão da informação para neurônios de segunda ordem. Essa transmissão resulta na percepção do estímulo e na transmissão da informação do estímulo periférico por meio de sinapses no córtex somatossensorial, fornecendo a localização e avaliação da intensidade do estímulo (Basbaum et al., 2009; Garland, 2012).

Com base nessas informações, a dor se apresenta não apenas como um dos sinais cardinais da inflamação, mas também como um complexo mecanismo capaz de intensificar o processo inflamatório. A compreensão dos mecanismos da dor é essencial não apenas para identificá-la como um sinal do processo

inflamatório, mas também como um mecanismo capaz de intensificá-lo, demonstrando assim a importância da resolução da inflamação.

1.4 Resolução da Inflamação.

A inflamação promove a eliminação do agente agressor na tentativa de restaurar a homeostase; no entanto, apesar de benéfica, pode se tornar prejudicial se intensa e prolongada (Chen et al., 2018), causando apoptose, necrose e até mesmo mutações celulares (Kay et al., 2019; Zindel e Kubes, 2020b). A resolução da inflamação é o mecanismo encarregado de esgotar o processo inflamatório através de uma série de eventos coordenados por uma gama complexa de mediadores que regulam os eventos celulares essenciais para a remoção das células inflamatórias dos locais de infecção ou lesão, restaurando a função dos tecidos (Buckley, Gilroy e Serhan, 2014).

Para uma resposta inflamatória ideal, é fundamental a autorregulação e controle que leve à sua resolução, à limpeza eficaz e à regeneração do tecido afetado, onde macrófagos e outras células do sistema imune desempenham um papel de destaque (Oishi e Manabe, 2018; Chawla, Nguyen e Goh, 2011). De modo geral, para promover a resolução eficaz da inflamação aguda, é essencial eliminar o estímulo desencadeador (Morgenstern et al., 1997) e a promoção da redução de mediadores pró-inflamatórios, como citocinas, quimiocinas, eicosanoides e moléculas de adesão celular (Charo e Ransohoff, 2006; Abbas, Pillai e Lichtman, 2019).

Além disso, a ingestão de corpos apoptóticos desencadeia a transformação de macrófagos fagocitantes pró-inflamatórios em fenótipos com características anti-inflamatórias, imunorregulatórias ou pró-resolução (Ariel e Serhan, 2012). Essa mudança de polarização desempenha um papel fundamental na preservação dos tecidos inflamados, minimizando os danos colaterais aos tecidos circundantes (Fadok et al., 1998).

O fenótipo pró-resolução é marcado pela regulação positiva de moléculas coibidoras, como o ligante de morte programada 1 (PDL1) e o ligante do

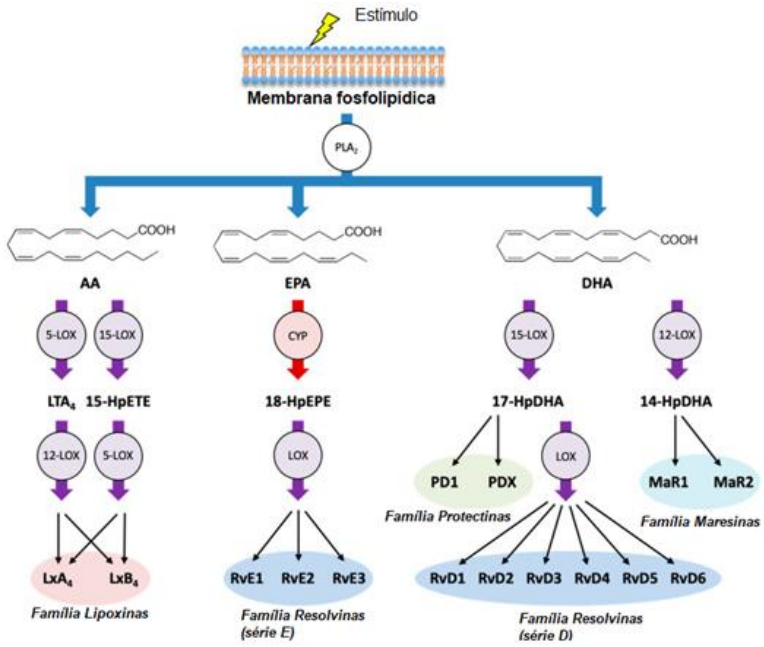
coestimulador induzível (ICOS), bem como a ativação de programas de transcrição genética anti-inflamatória, resultando na liberação de fator transformador de crescimento (TGF)-β, IL-10 e secreção de fatores associados ao antígeno nuclear proliferativo (PCNA), cAMP e PGE2 (Bystrom et al., 2008; Fadok et al., 1998). Os macrófagos que fagocitam células apoptóticas liberam Mediadores Lipídicos Pro-Resolução Especializados (SPMs), descritos como atores-chave na resolução da inflamação, como a Resolvina (Rv)E1, a Protectina D1 e as Maresinas 1 e 2, contribuindo assim para a resolução do processo inflamatório (Schwab et al., 2007; Serhan et al., 2012).

1.5 Mediadores Lipídicos Pro-Resolução Especializados (SPMs).

Os Mediadores Lipídicos Pro-Resolução Especializados (SPMs) agem como atores-chave na resolução da inflamação, auxiliando o influxo de granulócitos para o sítio de inflamação, fagocitose de patógenos e restos celulares, limitando a dor, ativando células residentes nos tecidos e impulsionando o seu reparo (Chiang et al., 2012; Fattori et al., 2020, 2021; Schett e Neurath, 2018; Serhan e Savill, 2005).

Os SPMs estão entre os principais responsáveis por mediar o processo ativo e coordenado da resolução da inflamação (Julliard et al., 2022). Derivados do metabolismo de ácidos graxos poliinsaturados (PUFAs), incluindo o ácido docosapentaenóico (DPA), ácido eicosapentaenóico (EPA), ácido araquidônico (AA) e ácido docosahexaenóico (DHA), são classificados em resolvinas, protectinas, lipoxinas e maresinas. São sintetizados por diversos tipos celulares, como fagócitos (principalmente macrófagos), células epiteliais e neurais (Julliard et al., 2022; Rasquel-Oliveira et al., 2023), como demonstrado a seguir (Figura 1).

Figura 1 – Vias de biossíntese dos mediadores lipídicos pró-resolução especializados (SPMs).



Fonte: Adaptado (Yasukawa, Okuno e Yokomizo, 2020).

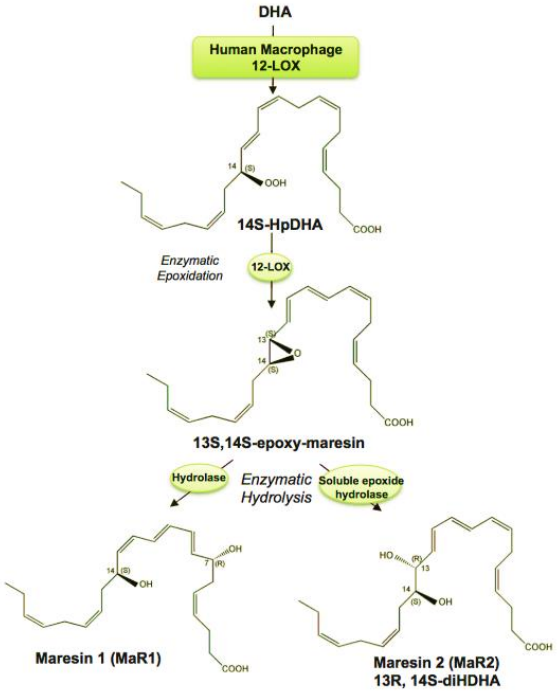
1.6 Maresina 2

As maresinas foram os últimos membros descritos da família dos SPMs e apresentam características anti-inflamatórias e pró-resolutivas. São sintetizadas por macrófagos a partir do DHA via 12-lipoxigenase humana (12-LOX) e são divididas em dois tipos distintos estruturalmente, mas apresentam atividades similares: maresina 1 (7R,14S-dihydroxy-docosa-4Z,8E,10E,12Z,16Z,19Z-hexaenoic acid) e maresina 2 (13R,14S-dihydroxy-4Z,7Z,9E,11E,16Z,19Z-hexaenoic acid (Deng et al., 2014).

As duas maresinas compartilham a mesma via inicial de síntese, que se inicia a partir da lipoxigenação do DHA pela 12-LOX, formando o DHA em 14S-hidroperoxiDHA (14S-HpDHA). Em seguida, uma segunda lipoxigenação é realizada pela mesma enzima, originando um intermediário epóxido, o 13S, 14S-epoxi-

maresina. O 13S, 14S-epoxi-maresina é convertido em 7R,14S-dihydroxy-docosa-4Z,8E,10E,12Z,16Z,19Z-hexaenoic acid (MaR1) por uma hidrolase, ou em 13R,14S-dihydroxy-4Z,7Z,9E,11E,16Z,19Z-hexaenoic acid (MaR2) por uma epóxido hidrolase solúvel (Deng et al., 2014; Ferreira et al., 2022), conforme demonstrado a seguir (Figura 2).

Figura 2 – Síntese de MaR1 e MaR2.



Fonte: Adaptado (Deng et al., 2014).

A MaR2 foi evidenciada e reconhecida por possuir capacidades anti-inflamatórias e pró-resolutivas, sendo capaz de reduzir a infiltração de leucócitos polimorfonucleares na cavidade peritoneal e aumentar a capacidade de fagocitose de macrófagos humanos (Deng et al., 2014). Além disso, ela reduz a inflamação alérgica das vias aéreas de camundongos, inibindo a ativação do inflamassoma NLRP3 (e consequente produção de IL-1β e IL-18 a) e o desenvolvimento de resposta imune do tipo Th2, além de diminuir o estresse oxidativo (Yu et al., 2022). A MaR2 encapsulada em nanopartículas de ácido polilático aumenta a migração epitelial e impulsiona o reparo da mucosa intestinal após colite (Miranda et al.,

2023). Ela também inibe a dor inflamatória dependente de TRPA1 e TRPV1 e a liberação de CGRP em camundongos, diminui o recrutamento de neutrófilos e a produção de mediadores pró-inflamatórios (CCL2, CCL5, CCL17, CCL20, CXCL2, CXCL10, MMP2, MMP9 e MPO) no tecido plantar de camundongos após injeção intraplantar de LPS. (Fattori et al., 2022). Além disso, demonstrou uma notável atividade analgésica tanto na dor inflamatória quanto na neuropática de origem orofacial. Essa atividade pode estar relacionada à inibição da ativação de neurônios CGRP-positivos (Lopes et al., 2023).

Portanto, a MaR2 possui notável potencial anti-inflamatório, pró-resolutivo e analgésico. A peçonha da *Bothrops jararaca* provoca inflamação e dor intensa nas vítimas, abrindo espaço para a importância do desenvolvimento de terapias complementares para aprimorar o manejo das complicações locais. Nesse contexto, a MaR2 emerge como uma molécula promissora, e até o momento, não existem estudos que tenham evidenciado o papel da maresina 2 como molécula anti-inflamatória e analgésica envolvendo a inflamação e dor causada pela peçonha de *Bothrops jararaca*.

2. OBJETIVOS

2.1. Objetivo Geral

Avaliar o efeito analgésico e anti-inflamatório da Maresina 2 na dor e inflamação induzida pela peçonha de *Bothrops jararaca* (BjV).

2.2. Objetivos Específicos

- Determinar a dose de BjV capaz de induzir hiperalgesia mecânica e térmica e a peritonite.
- Determinar a dose de MaR2 capaz de reduzir a hiperalgesia mecânica e térmica induzida por BjV.
- Avaliar o efeito do tratamento com MaR2 na distribuição de peso das patas traseiras, no recrutamento leucocitário, no estresse oxidativo e nos níveis de citocinas pró-inflamatórias no tecido plantar.
- Determinar a dose de MaR2 capaz de reduzir a peritonite induzida por BjV.
- Avaliar o efeito do tratamento com MaR2 sobre o recrutamento leucocitário e o estresse oxidativo na cavidade peritoneal.

3. CONCLUSÃO

Em resumo, nosso estudo esclarece o potencial terapêutico da Maresina 2 em casos de acidentes ofídicos envolvendo a *Bothrops jararaca*. Pela primeira vez, evidenciamos que o pré-tratamento com Maresina 2 reduz eficazmente a dor inflamatória e reduz diversos aspectos da resposta inflamatória aguda desencadeada pela peçonha. O pré-tratamento reduziu a migração de leucócitos, a produção de espécies reativas de oxigênio e de citocinas pró-inflamatórias, ao mesmo tempo em que fortalece as defesas antioxidantes. Nossos dados destacam a MaR2 como uma opção promissora para o tratamento de condições inflamatórias e dolorosas associadas a picadas de serpentes, abrindo novas possibilidades para futuras explorações no desenvolvimento de intervenções terapêuticas para esse problema crítico de saúde.

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Maresin 2 reduces pain and inflammation induced by *Bothrops jararaca* venom in mice.

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ABSTRACT:

Snake bites have the potential to trigger inflammation and pain. The venom of *Bothrops jararaca* (BjV) induces intense and prolonged pain that is resistant to antivenom, along with hemorrhage, inflammatory cell infiltration, and edema. Maresin 2 (MaR2) is a Specialized Pro-Resolving Lipid Mediator (SPM) with anti-inflammatory, pro-resolving, and analgesic properties. This study aimed to assess the efficacy of MaR2 in reducing pain and inflammation caused by BjV in mice. Mechanical hyperalgesia analysis (using the electronic version of von Frey filaments), thermal hyperalgesia (hot plate test), and weight distribution (static weight support) were conducted. Additionally, myeloperoxidase activity (MPO assay), superoxide anion (O2-) production (NBT assay), total antioxidant levels (ABTS assay), and levels of TNF-α, IL-1β, and IL-6 cytokines in the plantar tissue of mice were evaluated after intraplantar injection of different doses of BjV (0.01, 0.1, and 1 μg). Intraperitoneal pre-treatment with MaR2 (0.3, 1, and 3 ng) demonstrated a dose-dependent reduction in mechanical and thermal hyperalgesia, normalization of weight distribution, inhibition of MPO activity, decreased O2- production, and increased total antioxidant levels in the tissue. Furthermore, a reduction in TNF-α, IL-1β, and IL-6 levels was observed. In a peritonitis model induced by intraperitoneal injection of different doses of BjV (1, 3, and 5 μg), leukocyte recruitment, hemorrhage, levels of reactive oxygen species (ROS) and intracellular nitric oxide (NO), O2- production, and the expression of gp91phox and inducible nitric oxide synthase (iNOS) mRNA were assessed using RT-qPCR. Intraperitoneal pre-treatment with MaR2 (0.1, 1, and 10 ng) resulted in a dose-dependent reduction in leukocyte recruitment, hemorrhage, ROS, NO, and O2- levels, as well as in the expression of gp91phox and iNOS mRNA. In summary, using models of inflammatory pain in the paw and peritonitis, this study suggests that MaR2 holds potential as a mediator for the treatment of pain and inflammation induced by BjV.

Keywords: Maresin 2; *Bothrops jararaca* venom; Pain; Inflammation.

1. Introduction

Snakebites are a health problem in several tropical and subtropical countries. Due to the significant social and economic impact of such accidents, in June 2017, during the 10th meeting of the Strategic and Technical Advisory Group for Neglected Tropical Diseases in Geneva, the WHO classified snakebite envenoming as Category A of Neglected Tropical Diseases [1]. Among the most affected countries, Brazil stands out with the highest number of cases in South America [1], accounting for approximately 69.3% associated with the Bothrops jararaca snake [2].

Understanding the bothropic venom pathogenesis is challenging due to the several pharmacologically active peptides and proteins found in its composition that can initiate a wide range of signs and symptoms observed in the victims. Local manifestations are mainly attributed to serin proteases, metalloproteases, and to phospholipase A2 [3], [4]. After the injury caused by the bite of the *Bothrops jararaca* snake, the site of the injury exhibits prolonged and intense pain [5], [6] that cannot be reversed by the antivenom [7], dermonecrosis, myonecrosis [3], [8], hemorrhage [9], inflammatory cells infiltrate [6], and edema [10] are also commonly found outcomes.

Leukocyte migration in response to venom-induced injury during the snakebite leads to the release of pro-inflammatory cytokines and excessive production of reactive oxygen species (ROS) [11], [12]. ROS, such as superoxide and nitric oxide, damage lipids, proteins, and nucleic acids in cells, resulting in cellular and tissue damage, contributing to the progression of inflammation [13].

Treatment for the *Bothrops* snake venom poisoning is based on intravenous injection of bothropic antivenom [14], which neutralizes its systemic actions but presents low effectiveness against the symptoms on the site of the injury [15]. Therefore, medications such as anti-inflammatories, pain killers, and antibiotics are commonly associated with the bothropic antivenom, even though they can add to the toxicity and initiate adverse reactions [16].

Considering the potential advantages associated with a novel alternative, the growing evidence that specialized pro-resolution mediators (SPMs) may represent a promising therapeutic alternative for the management of inflammation and pain [17], [18]. The components of lipid mediators include several substances as examples: lipoxins, resolvins, maresins, and protectins [17], [18]. Among SPMs, we can highlight Maresin 2 (MaR2), a lipid mediator derived from the docosahexaenoic acid (DHA) fatty acid [19]. MaR2 has garnered attention due to its anti-inflammatory, pro-resolutive, and analgesic attributes [19]–[23]. As the venom of the *Bothrops jararaca* snake (BjV) is known to induce inflammation and severe pain, this study seeks to assess the effectiveness of MaR2 in reducing the pain and inflammation provoked by BjV in mice.

2. Materials and methods

2.1. Animals

Swiss female mice (25-30 grams) were provided from Londrina's State University (Londrina, PR, Brazil), allocated in clear standard plastic cages with free access to food and water, and submitted to 12 hours light/dark cycles and constant 21°C temperature. For all behavior experiments, mice were previously acclimated to

the laboratory for at least two days during one-hour long adaptions to the experimental cages. All steps followed IASP (International Association for the Study of Pain) guidelines and were sanctioned by the Ethics Committee of Animal Experimentation from Londrina's State University, office number 103/2021.

2.2. General Experimental Procedures

For the first series of experiments, dose-responses curves to both the stimulus with BjV and the treatment with MaR2 were conducted to determine the ideal doses for the following experiments. Female Swiss mice right hind paw (i. pl.) was injected with different doses of BjV (0.01, 0.1 and 1 µg/paw). Mechanical and thermal hyperalgesia were assessed 1, 3, 5 and 7 hours after BjV stimulus, and daily for the next four days. The dose of 1 µg/paw of BjV was elected for the following experiments on paw. Next, a dose-response to determine MaR2's optimal dose was conducted with intraperitoneal (i.p.) injection of 0.3, 1 or 3 ng/cavity 30 minutes prior to BjV stimulus, and thermal and mechanical hyperalgesia tests were performed. The dose of 3 ng/cavity was elected based on the results obtained. Additionally, the behavior teste Static Weight Bearing was conducted to detect weight distribution shifts between hind paws.

To evaluate possible antioxidant and anti-inflammatory effects of MaR2 (pre-treatment 30 min prior to stimulus), plantar paw tissue was collected 7 hours after BjV i.pl. injection and MPO, NBT, ABTS oxidative assays were performed, as well as pro-inflammatory cytokines quantitation of TNF-α, IL-1β and IL-6.

For the second set of experiments, an intraperitoneal BjV dose-response was conducted with 1, 3 or 5 µg/cavity. Leukocyte recruitment was analyzed 15 hours after BjV injection, and the dose of 3 µg was elected based on total and differential leukocyte recruitment results. A dose-response for MaR2 treatment was carried out as well with 0.1, 1 or 10 ng/cavity. The elected dose based on differential and total leukocyte recruitment results was 1 ng/cavity. Total erythrocytes count on peritoneal cavity was also performed, followed by ROS and NO activity by probe fluorescence intensity investigation. Superoxide anion reduction was evaluated on NBT assay and expression of gp91Phox and iNOS ere verified by RT-qPCR. All experiments were analyzed by investigators blinded to the groups.

2.3. Test Compounds

Drugs and compounds used were: Maresin 2 (13R,14S)-dihydroxy-(4Z,7Z,9E,11E,16Z,19Z)-docosahexaenoic acid; Cayman Chemicals, Ann Arbor, Michigan, EUA), *Bothrops jararaca* venom (BjV; Laboratory of Immunopathology Laboratório de Imunopatologia, Butantan Instititute, SP, BR), saline solution (NaCl 0,9%; Fresenius Kabi Brasil Ltda. Aquiraz, CE, BR).

2.4. Mechanical hyperalgesia assessment

Mechanical hyperalgesia test Mechanical hyperalgesia was performed by the electronic von Frey anesthesiometer (Insight, Ribeirão Preto, SP, BR), evaluated as previously described [33]. Briefly, mice (n=6 per group) were placed in acrylic cages (12 X 10 × 17 cm) with wire grid floors, in a quiet room for at least 1 h before the test for environmental adaptation. The test consists of evoking a hind paw flexion reflex with a hand-held force transducer adapted with a 0.5 mm2 contact area polypropylene tip. Then, the experimenter applies the probe perpendicularly to the central area of the hind paw with a gradual increase of pressure. The endpoint was characterized by the removal of the hindlimb followed by clear flinch movements. The measurements were standardized to be always measured on the right paw of mice. The electronic pressure-meter apparatus automatically recorded the intensity of the pressure applied when the paw was withdrawn. The value for the response was an average of three measurements. The results are expressed by delta (Δ) withdrawal threshold (basal-time mean measurements were subtracted from mean measurements after BjV injection at 1, 3, 5, and 7 h, and daily throughout the next 4 days).

2.5. Thermal Hyperalgesia assessment

Thermal hyperalgesia was performed by hot plate test (Insight, Ribeirão Preto - SP, Brazil) at 52ºC ±1 ºC. In a quiet room, mice (n=6 per group) were positioned individually on a heated surface of the hot plate apparatus isolated by containment recipient of transparent acrylic material. The reaction time was registered using a conventional chronometer when the mice presented the behaviors of licking or flinching one of the hind paws. A time limit of 15 s of maximum latency was defined as the cut-off, with the intention of avoiding potential tissue injuries. The assessment of thermal hyperalgesia was performed before (baseline T0) and after the BjV injection (at 1, 3, 5, and 7 h, and daily throughout the next 4 days). The results were expressed by the thermal threshold in seconds (s).

2.6. Static Weight Bearing (SWB)

Frequently, inflammation leads to tissue damage that can initiate both central and peripheral sensitization which, in turn, results in significative changes in weight distribution as it is directed towards the non-affected limber [34]. To evaluate such alteration, a Static Weight Bearing (Bioseb, Vitrolles, France) device was used. The animals were individually conditioned in an acrylic device positioned forward, with each mouse’s front paws supported at the front, while their hind legs were supported by a weight measurement sensor (g). The test began in the moment the animal was immobilized; then, weight measurement was performed for 10 s. At the end of this period, the weight of the left and right paw was measured, with the right being relative to the stimulus. This test evaluates the weight distribution between a mouse’s hind legs. While an unstimulated animal distributes its weight equally between the two legs, the ratio of weight distribution between the stimulated and unstimulated paw is a measure of the level of discomfort in the stimulated paw. The mean of two measurements were recorded before BjV stimulus (T0), 1, 3, 5, and 7 hours after stimulus, and daily for four days after venom administration. Results are presented as right to left paw pressure ratio.

2.7. Evaluation of Leukocyte Migration and Profile

Bothrops jararaca snake venom induces long-lasting leukocyte migration to the peritoneal cavity [35]. To evaluate immune cell recruitment, BjV was injected intraperitoneally, and exudate was collected15 hours later. 5 mL of iced Phosphate-Buffered Saline (PBS) (1x), pH 7.0, with EDTA 2 mM were injected into the peritoneum to obtain recruited leukocytes. 20 µL of exudate were diluted in 180µL of Turk solution (to lysate erythrocytes), and total leukocyte counts was performed with a Neubauer chamber. Differential leukocyte count was carried out on microscopy slides prepared using a cytocentrifuge (Shandon Cytospin 3, Tokyo, Japan), stained with fast panoptic (Laborclin, Pinhais, Brazil), and analyzed with brightfield microscopy (Olympus CX31RTSF, Tokyo, Japan). Results were expressed as total leukocyte count (cells × 10^6 /cavity) and percentage (%) of polymorph and mononuclear cells.

2.7.1 Myeloperoxidase activity (MPO)

Mice plantar paw tissue neutrophil migration was evaluated by the colorimetric detection of myeloperoxidases, as previously described [36]. Samples were collected 7 hours after intraplantar injection with on iced K2HPO4 (50 mM) buffer (pH 6,0) with 0,5% HTAB, and kept frozen at -80°C. Briefly, samples were homogenized and centrifuged (14000 rpm × 2 min). 5 µL of the supernatant were added to 200 µL of phosphate buffer (50 mM), pH 6.0, with 0,167 mg/mL of o-dianisidine dihydrochloride and 0,015% hydrogen peroxide. The absorbance was measured at 600nm after 20 min (Multiskan GO Microplate Spectrophotometer, ThermoScientific, Vantaa, Finland). The MPO activity of the sample was compared to a standard curve of neutrophils. Results are presented as MPO activity (number of neutrophils × 10^3 per milligram of tissue.

2.8. Oxidative stress

2.8.2. Nitroblue Tetrazolium reduction (NBT) assay

Superoxide anion production on paw tissue homogenate and peritoneal cells wash were assessed through NBT assay, as previously described [36]. Plantar paw tissue or peritoneal wash were collected 7 hours after BjV i.pl. or i.p. induction, respectively, and mechanically disrupted. In sum, 100 µL of tissue homogenate or peritoneal wash were added to 100 µL of NBT 1mg/mL on a 96 well plate for one hour. Supernatant was removed and precipitated formazan crystals were solubilized adding 120 µL of KOH 2 M and 120 µL of dimethyl sulfoxide (DMSO). NBT reduction was measured on 600nm by spectrophotometry Microplate Spectrophotometer (Multiskan GO, ThermoScientific, Vantaa, Finland). Results from tissue samples were presented as optical density per milligram of tissue, whereas results from peritoneal wash were presented as optical density per µg of protein.

2.8.3. Microscopic NBT-Positive Cell Count

Production of superoxide anion was evaluated by the deposition of formazan crystals in the cytoplasm and cellular membrane of leukocytes. The reduction of NBT from a light-yellow dye into a precipitated dark blue crystal (formazan) is observed under light microscopy [37]. For the microscopic assessment of NBT-positive cells, the peritoneal exudate was collected 7 hours after the BjV stimulus. 50 µL of peritoneal wash were incubated with 50 µL of NBT (1 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 30 minutes. Microscopy slides were prepared using a cytocentrifuge (Shandon Cytospin 3, Tokyo, Japan), stained with fast panoptic (Laborclin, Pinhais, Brazil), and the number of NBT-positive cells was counted under light microscopy (Olympus CX31RTSF, Tokyo, Japan). The values are expressed as a percentage of NBT-positive cells.

2.8.4. Antioxidant efficiency by radical sequestration assay 2,2´azinobis (3-sulfonic ethylbenzothiazoline-6 acid) (ABTS)

Oxidative damage resistance was investigated through the efficiency on eliminating free radicals observed by ABTS assay [38]. Plantar paw tissue was collected 7 hours after BjV stimulus, homogenized in 500 µL of KCl (1.15%) using a Tissue-Tearor (Biospec 985370), centrifuged at 1000 g for 10 min at 4°C and the supernatant was used to measure the antioxidant efficiency of MaR2. The solution of ABTS was prepared with 7 mM of ABTS and 2.45 mM of potassium persulfate diluted with phosphate buffer pH 7.4 to an absorbance of 0,7 – 0,8 in 730 nm was prepared. The supernatant was mixed on ABTS solution and after 6 min the absorbance was determined in a 730 nm microplate reader (Multiskan GO, ThermoScientific, Vantaa, Finland). Previously, a standard Trolox curve (0,01 a 20 nmol) was prepared, and the results were expressed as equivalent nmol of Trolox/mg skin.

2.8.5. Total Intracellular ROS Detection

Production of intracellular ROS was evaluated using an H2DCFDA probe (ID: D99, H2-DCF, Invitrogen, Massachusetts, USA). Peritoneal exudate wash was collected 15 hours after i.p. injection of BjV on Facs Buffer (PBS [152 mM], [pH 7.0], EDTA [2 mM] and 0,5% Bovine Serum Albumin [BSA]). Samples were centrifuged (1.200 rpm, 10 min), supernatant were discarded, and cell pellets were seeded on microscopic glass bottom culture plates using incomplete RPMI medium. Cells were kept in CO2 incubator (37°C, CO2 5%) for 30 min to adhere. Probe H2DCFDA 20 mM (1:200) was added for incubation for another 40 min, plates were washed three times with HBSS, and cells were analyzed by confocal microscopy (TCS SP8, Leica, Mannheim, Germany), objective of 63 × on 488 wavelength fluorescence using Software Leica X (LAS X, Leica, Mannheim, Germany) to obtain images. Analysis was performed by investigators blinded to the experimental groups.

2.8.6. Intracellular Nitric Oxide (NO) Detection

Intracellular nitric oxide levels were assessed using fluorescence microscopy with DAF-FM (D23842, Invitrogen, Massachusetts, USA) probe. Cells was collected and procedures were carried out as described on item 2.8.5 until the point of probing.

For NO detection, DAF-FM (1:500) probe was added, plates were washed three times after 40 min of incubation, and images were obtained by confocal microscopy (TCS SP8, Leica, Mannheim, Germany), objective of 63 × on 488 wavelength fluorescence using Software Leica X (LAS X, Leica, Mannheim, Germany) to obtain images. Analysis was performed by investigators blinded to the experimental groups.

2.9. Cytokines quantitation

Mice plantar paw tissue was collected 7 hours after intraplantar injection of BjV and frozen at -80°C. Briefly, samples were homogenized using an Ultra Turrax® (T18 basic, IKA) on 500µL of saline solution 0,9% and centrifuged (3.000 RPM × 15 min × 4°C). Supernatant were collected to determine cytokines TNF-α, IL-1β and IL-6 levels using ELISA commercial kits following manufacturers' instructions (DuoSet ELISA Development [R&D Systems, DY410; DY401] e ELISA MAX™ Deluxe Set Mouse IL-6 [Biolegend, 431301], respectively). Results are presented as picograms per mg of plantar paw tissue.

2.10. Erythrocytes Count

Metalloproteinases present in snake venom are known to generate a series of symptoms, mainly related to local or systemic hemorrhage [36]–[38]. To investigate hemorrhage in peritoneal cavity after BjV induction, exudate was collected according to the procedures followed on total and differential leukocyte counts assays, but instead of using Tukey solution, 180µl of Dacie solution, used to lyse leukocytes, was added to 20µl of peritoneal exudate. Erythrocyte count was performed using a Neubauer chamber. Results are expressed as total erythrocytes ×10^3 per cavity.

2.11. RT-qPCR

Peritoneal exudates were collected 7 hours after i.p. injection of BjV. The abdominal cavity was washed once with 5mL of sterile saline with EDTA 2mM. Cells were lysed (QIAzol Lysis Reagent, QIAGEN, ID: 79306) and total RNA was extracted following manufacturer instructions. RNA purity and concentration were measured by UV spectrophotometry (Multiskan GO Microplate Spectrophotometer, Thermoscientific, Vantaa, Finland) (260/280nm) adopting purity cutoff between 1,8 and 2,0. Reverse transcription was carried out using QuantiTect Reverse Transcription Kit (400) (QIAGEN, 205314) and for qPCR the QuantiNova SYBR Green PCR Kit (QIAGEN, 208056). Readings were performed using StepOnePlusTM Real-Time PCR System (Applied Biosystems ®, Waltham, MA, EUA). Relative gene expression was obtained through the comparative method 2−(ΔΔCt). Primers sequences used on this analysis are detailed on table 1. The constitutive Gapdh gene was used to normalize data.

Gene	Sense	Antisense	Manufacturer and prime number

Gapdh	5'-GCCCAGAACATCATCCCTGC-3'	5'-GCCTCTCTTGCTCAGTGTCC-3'	Invitrogen Sense: I3932A11 Antisense: 13932A12
Gp91phox	5'-AGCTATGAGGTGGTGATGTTAGTGG -3'	5'-CACAATATTTGTACCAGACAGACTTGAG-3'	Invitrogen Sense: I2203C11 Antisense: I2203C12
Inos	5'-CGAAACGCTTCACTTCCAA-3'	5'-TGAGCCTATATTGCTGTGGCT-3'	Invitrogen Sense: I2203B07 Antisense: I2203B08

Table 1. Primer sequences used in RT-qPCR.

2.12. Statistical Analysis

Data were analyzed using the software GraphPad Prism (GraphPad Software, version 9.0.0). Results are presented as mean ± SEM or median with range and samples normal distribution was verified through Shapiro-Wilk's test. Normally distributed data was analyzed with one or two-way ANOVA (according to the presence or absence of multiple time-points) followed by Tukey. Data without normal distribution was analyzed with Kruskal-Wallis' non-parametric test followed by Dunn. Groups were considered statistically different if $p \leq 0,05$.

3. Results

3.1. MaR2 inhibits BjV-induced inflammatory pain.

Following the treatment protocol (Fig. 1A), a dose-response with 0.01, 0.1 and 1µg of BjV injection in the right hind paw was conducted to determine the ideal stimulus rate. Based on mechanical and thermal hyperalgesia assays, the dose of 1µg/paw was elected (Fig. 1B-C) to investigate the analgesic effect of MaR2.

To do so, mechanical, and thermal hyperalgesia dose-responses were conducted with intraperitoneal injections of 0.3, 1 or 3 ng/cavity of MaR2 30 minutes before stimulus with BjV (Fig. 1A). Saline groups indicate absence of hyperalgesia in both von Frey and hot plate assays. BjV induced mechanical hyperalgesia in all observed time-points, while thermal hyperalgesia was present until the 7th hour (Figures 1D-E). Meanwhile, treatment with 1ng of MaR2 reduced mechanical hyperalgesia on the first day, and the 3ng dose was able to prolong this effect until the second day of measurement (Fig. 1D). Thermal hyperalgesia was inhibited from the 3rd until the 7th hour (Fig. 1E). Therefore, the 3ng dose was elected for the following experiments.

To investigate hyperalgesia from a non-evoked approach, changes in weight bearing distribution of the hind paws were observed. BjV injection reduced right to left

paw ratio distribution from the 1st until the 7th hour (Fig. 1F-G), while the group previously treated with MaR2 returned to baseline measurements from the 3rd hour on, indicating its analgesic potential (Fig. 1F-G).

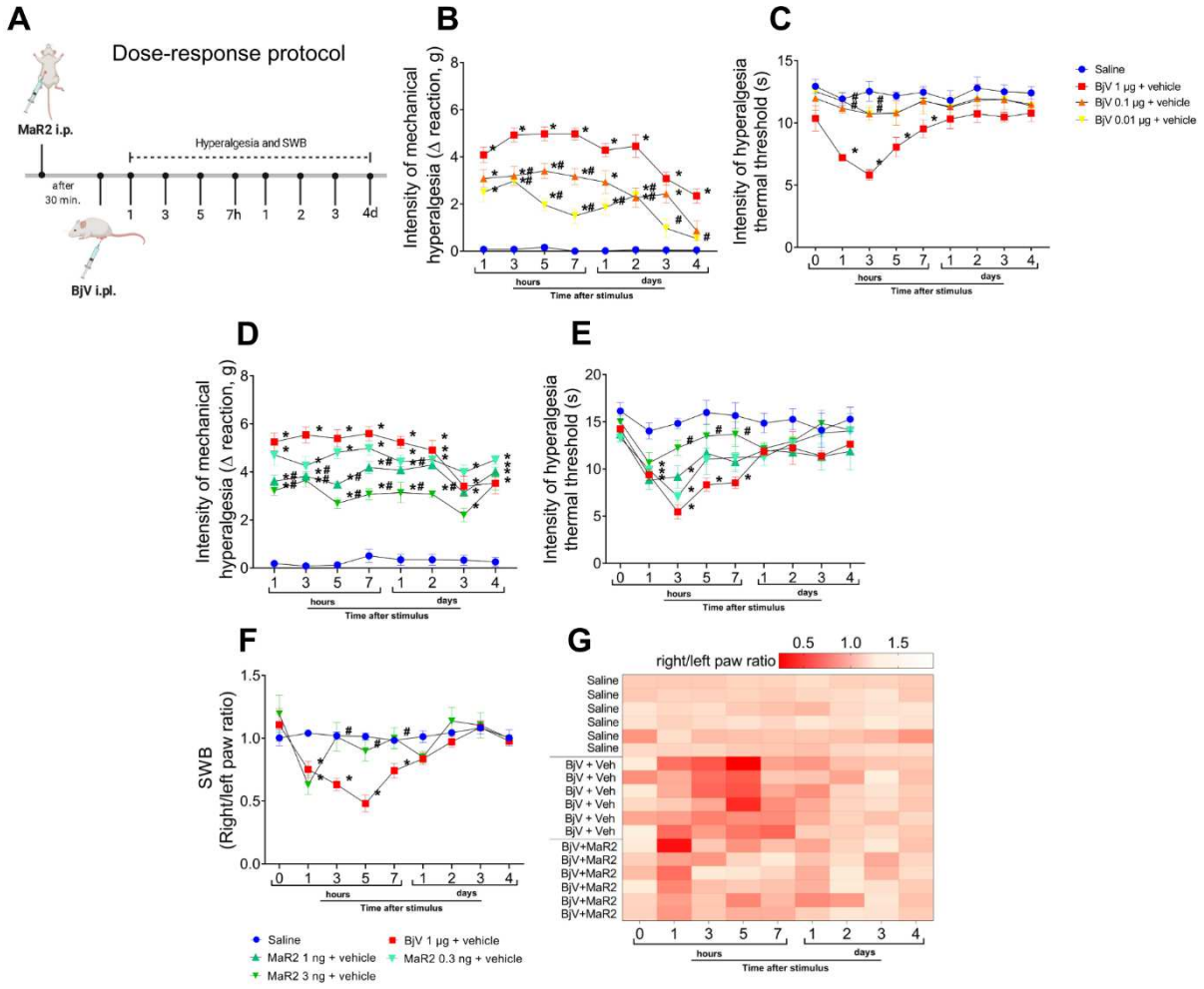

Figure 1. MaR2 reduces inflammatory pain induced by BjV. Dose–response treatment protocol scheme (A). Mechanical (B) and thermal (C) hyperalgesia were evaluated after 1, 3, 5 and 7 hours, and daily for 4 days after BjV injection (1, 0.1 e 0.01 µg/paw, i.pl.) through von Frey and hot plate assays, respectively. MaR2 dose-response (0.3, 1 e 3 ng/cavity, i.p.) 30 minutes before BjV injection (1 µg/paw, i.pl.) was conducted through the same techniques (D e E). Results are presented as Δ means (B and D); means (C and E) ± SEM; n = 6 mice per group (* P < 0,05 vs saline; $^{\#}P$ < 0,05 vs BjV [two-way ANOVA followed by Tukey]). SWB was elected as a non-evoked approach to evaluate sensitivity to pain (F). The heatmap demonstrates the ratio between right and left hind paws of each mouse in different time-points (G). Results are presented as the ratio between right and left hind paws of each mouse; n = 6 mice per group (* P < 0,05 vs saline; $^{\#}P$ < 0,05 vs BjV 1 µg + vehicle [two-way ANOVA followed by Tukey]).

3.2. MaR2 reduces neutrophil infiltration, oxidative stress, and levels of TNF-α, IL-1β, and IL-6 in the paws of mice injected with BjV.

On the early stages of the acute inflammatory response, polymorphonuclear leukocytes (PMNs), essentially neutrophils, are recruited to post-capillary venules to initiate phagocytosis of microorganisms and cellular debris [30]. An overreaction of

PMNs in inflamed tissue intensifies the inflammatory response, resulting in tissue damage [31].

The i.pl. injection of BjV caused an increase in neutrophil recruitment to the plantar tissue, as revealed by the indirect quantification of these cells through the measurement of myeloperoxidase (MPO) presence, the elevation of superoxide anion production, and the reduction of total antioxidants. Additionally, there was an increase in the levels of the cytokines TNF-α, IL-1β, and IL-6 in the paw tissue.

The i.pl. injection of BjV caused an increase in neutrophil recruitment to the plantar tissue, as revealed by the indirect quantification of these cells through the measurement of myeloperoxidase (MPO) presence, the elevation of superoxide anion production, and the reduction of total antioxidants (Fig. 2A-C). Additionally, there was an increase in the levels of the cytokines TNF-α, IL-1β, and IL-6 in the paw tissue (Fig. 2D-F).

Treatment with MaR2, on the other hand, was able to reduce neutrophil recruitment (Fig. 2A), and to inhibit superoxide anion production (Fig. 2B). We also observed an increase in total antioxidant levels (Fig. 2C) and reduced TNF-α, IL-1β, and IL-6 cytokine levels (Fig. 2D-F) on mice paw tissue.

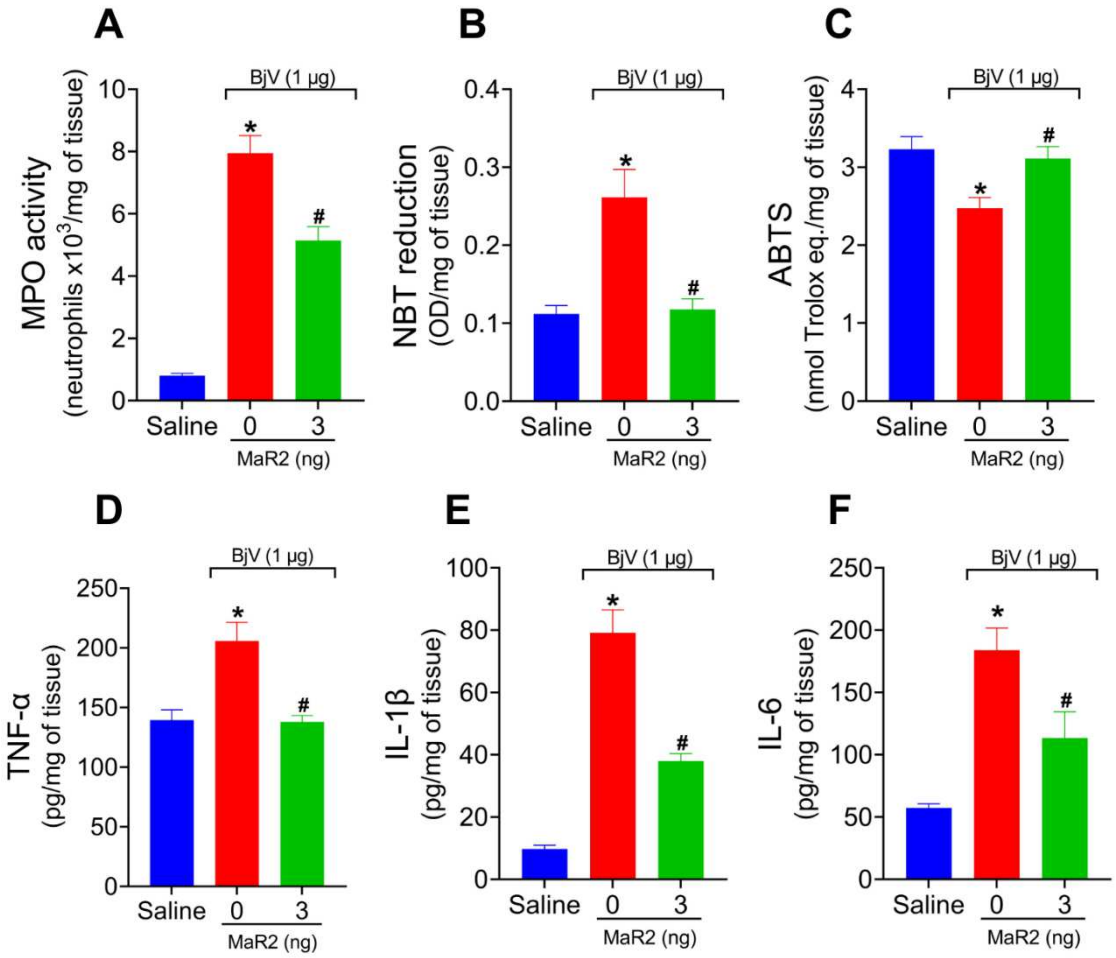

Figure 2. MaR2 reduces neutrophil infiltration and superoxide anion production, increases antioxidant properties, and downregulates pro-inflammatory cytokine levels. Mouse paw tissue was dissected 7 hours post-injection with BjV (1 µg/paw, i.pl.) to determine MPO levels, superoxide anion production (A and B), and total antioxidant properties (C, ABTS assay) through colorimetric techniques. Levels of cytokines TNF-α, IL-1β, and IL-6 (D-F) were assessed immunoenzymatically via ELISA. Results are presented as mean ± SEM; MPO assay n = 12 mice per experimental group; NBT assay n = 6 mice per group (*P < 0.05 vs. saline; #P < 0.05 vs. 0 [One-way ANOVA followed by Tukey]).

3.3 MaR2 inhibits leukocyte recruitment, hemorrhage, and oxidative stress in peritonitis induced by BjV.

3.3.1. MaR2 inhibits leukocyte recruitment.

Following the treatment protocol (Fig. 3A), different doses of BjV were tested (1, 3 e 5 µg/cavity) to determine ideal stimulus for 15 hours (Fig. 3B-D). To establish if MaR2 could reduce leukocyte recruitment, a dose-response curve with 0.1, 1 e 10 ng/cavity injected intraperitoneally 30 minutes before BjV intraperitoneal (Fig. 3E). Peritoneal cells wash was also collected 15 hours after BjV injection (Fig. 3E-G). As BjV injection increased total leukocyte recruitment, MaR2 treatment was effective to inhibit recruitment in all doses tested (Fig. 3E).

Accordingly, all doses reduced mononuclear cells (Fig. 3F). Furthermore, doses of 1 and 10 ng were able to reduce the number of neutrophils in the peritoneal cavity (Fig. 3G). Therefore, MaR2 decreased BjV-induced inflammatory response on mice peritoneal cavity. Based on these results, the dose of 1 ng/cavity of MaR2 was elected for the following experiments on peritoneal cavity.

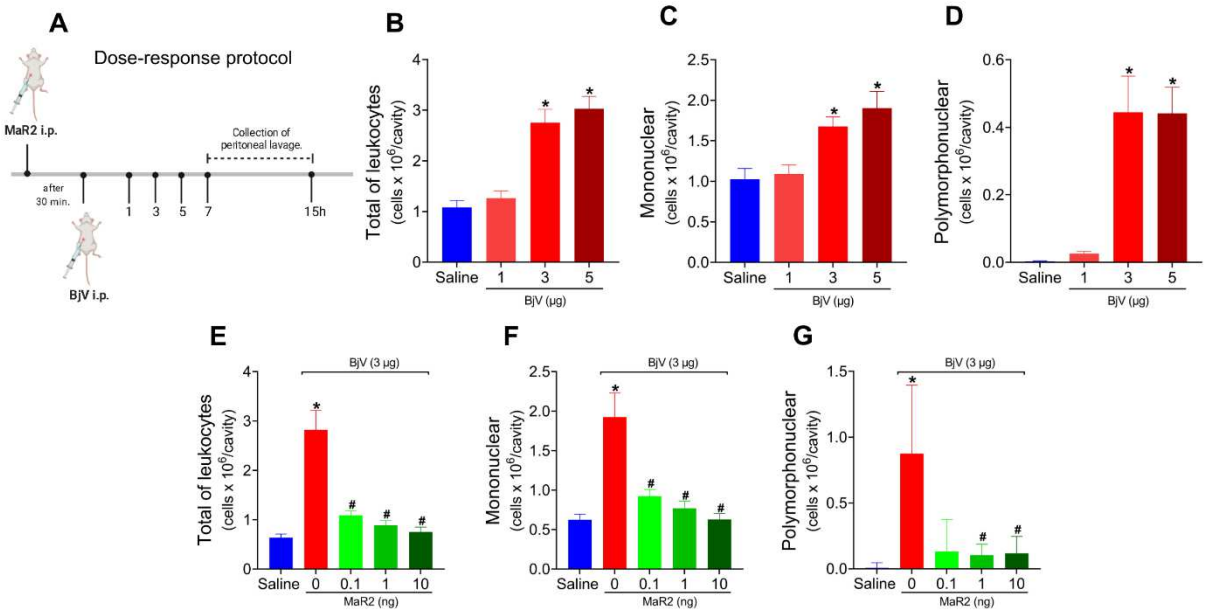

Figure 3. MaR2 inhibits leukocyte recruitment on peritoneal cavity induced by BjV. Dose–response treatment protocol scheme (A). BjV dose-response (1, 3 and 5 µg/cavity, i.p.) for 15-hour peritoneal injection was conducted (B-D). Results are presented as mean ± SEM; n = 8 mice per group (*p < 0,05 vs saline [one-way ANOVA followed by Tukey]). A dose-response was also conducted to determine MaR2's optimal dose (0.1, 1 and 10 ng/cavity, i.p., injected 30 minutes before BjV induction) (E-G). Results are presented as mean ± SEM (E and F) and median with range (G); n = 8 mice per group (* p < 0,05 vs saline; # p < 0,05 vs group 0; for total leukocyte and mononuclear cells assessment [one-way ANOVA followed by Tukey]; and polymorphonuclear cells [Kruskal-Wallis followed by Dunn's]).

3.3.2. MaR2 inhibits hemorrhage.

Local hemorrhage is a trait result of bothropic poisoning, and it is caused by the activity of metalloproteinases present in the snake venom (SVMPs) of Bothrops jararaca [32]–[34]. Mice induced with BjV showed high concentration of erythrocytes in the peritoneal cavity, while pre-treatment with MaR2 reduced the effects of BjV, demonstrating its anti-hemorrhage property in this model (Fig. 4).

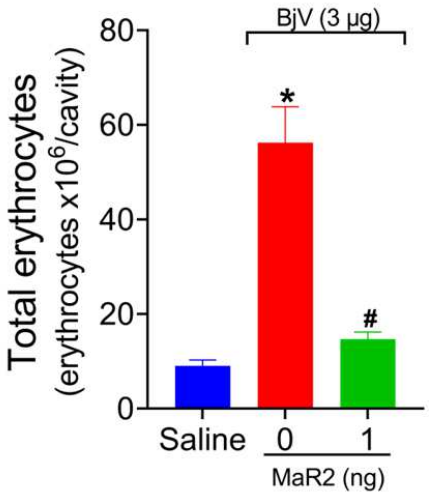

Figure 4. MaR2 inhibits BjV-induced hemorrhage. Peritoneal cell wash was collected 15 hours after BjV injection (3 µg/cavity, i.p.) to analyze total leukocyte count and total erythrocyte count. Results are presented as mean ± SEM; n = 8 mice per group (*p < 0,05 vs saline; #p < 0,05 vs group 0 [one-way ANOVA followed by Tukey]).

3.3.3. MaR2 inhibits leukocyte activity and oxidative stress.

It's been established that MaR2 can reduce oxidative stress [20]. The next step of the present work was to confirm whether it could reduce stress in peritonitis model induced by BjV assessing total reactive oxygen species (ROS) levels and intracellular nitric oxide (NO), superoxide anion production and mRNA expression of gp91phox, a subunit of NADPH oxidase, an enzyme related to the superoxide anion production, [35], and nitric oxide synthase (iNOS), a crucial enzyme in the production of nitric oxide [36].

MaR2 reduced both DAF and DCF fluorescence production, which means it was able to decrease total ROS production and intracellular NO (Fig. 5 A and B; 6 A and B). Additionally, it was able to reduce the mRNA expression of gp91phox and iNOS (Figure 5C and 6C, respectively), the superoxide anion production in peritoneally recruited leukocytes (Figure 5D), the quantity of leukocytes positive for NBT (Figure 5E and F), indicating that it was able to decrease the production of reactive oxygen species in the recruited cells.

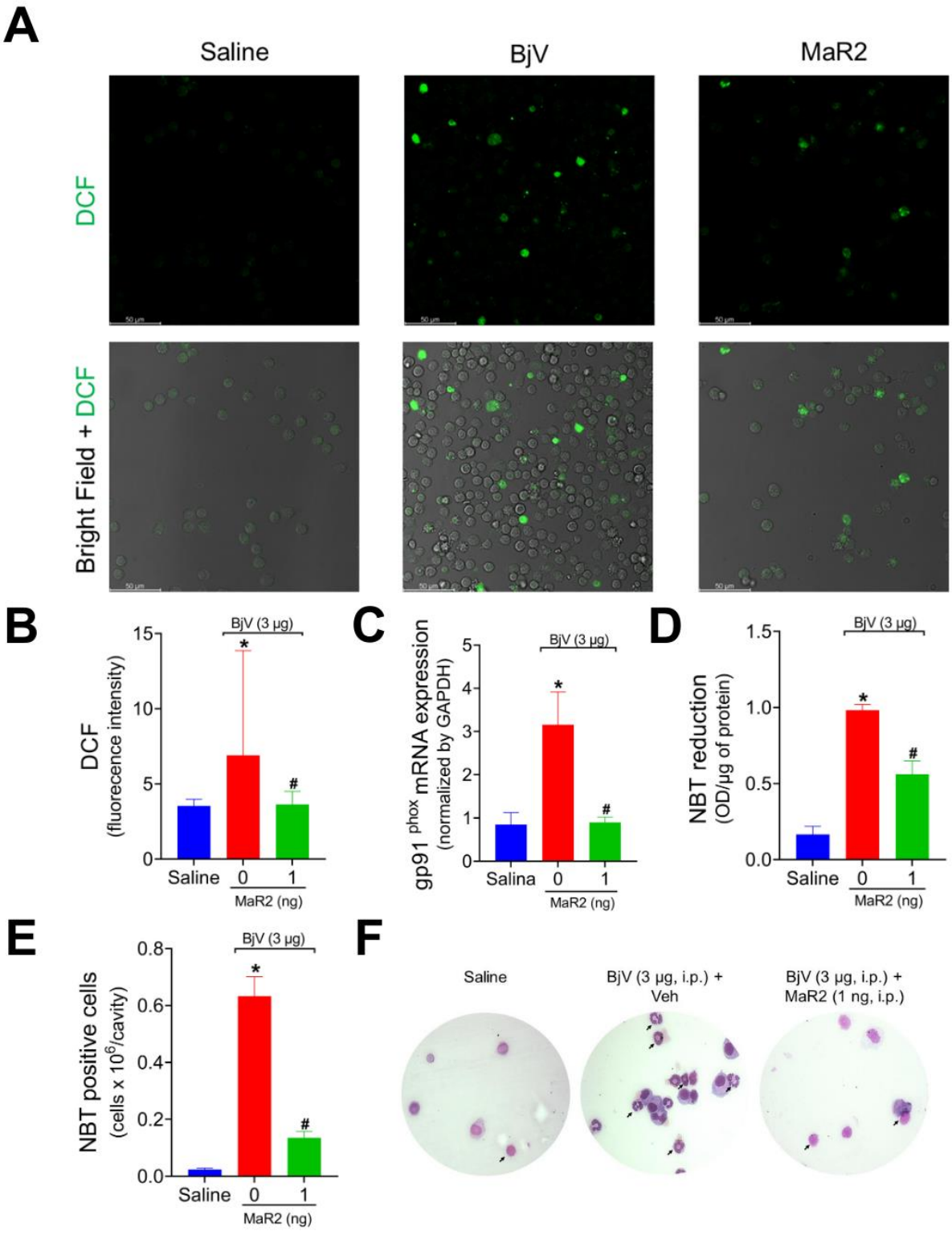

Figure 5. MaR2 reduces intracellular ROS, expression of gp91phox and superoxide anion production on BjV-induced peritonitis model. Levels of intracellular ROS were observed with H2-DCF probes on brightfield and 488nm channel with 63x amplification on confocal microscope (A and B). Effects of MaR2 on superoxide anion production (D, NBT assay) and NBT-positive cell count (E and F) on peritoneal exudate were evaluated 15 hours post BjV stimulus. DCF assay: n = 10 mice per group (*p < 0,05 vs saline; #p < 0,05 vs group 0 [Kruskal-Wallis followed by Dunn's]); NBT and formazan: n = 6 mice per group (*p < 0,05 vs saline; #p < 0,05 vs group 0 [one-way ANOVA followed by Tukey]). gp91phox mRNA expression (C) was evaluated 7 hours after BjV peritoneal stimulus. Relative gene expression was measured using comparative 2−(ΔΔCt) with Gapdh as constitutive

gene. gp91phox mRNA expression: n = 5 mice per group (* P < 0,05 vs saline; # P < 0,05 vs group 0 [one-way ANOVA follow by Tukey]). Results are presented as mean ± SEM (C-E) and median with range (B).

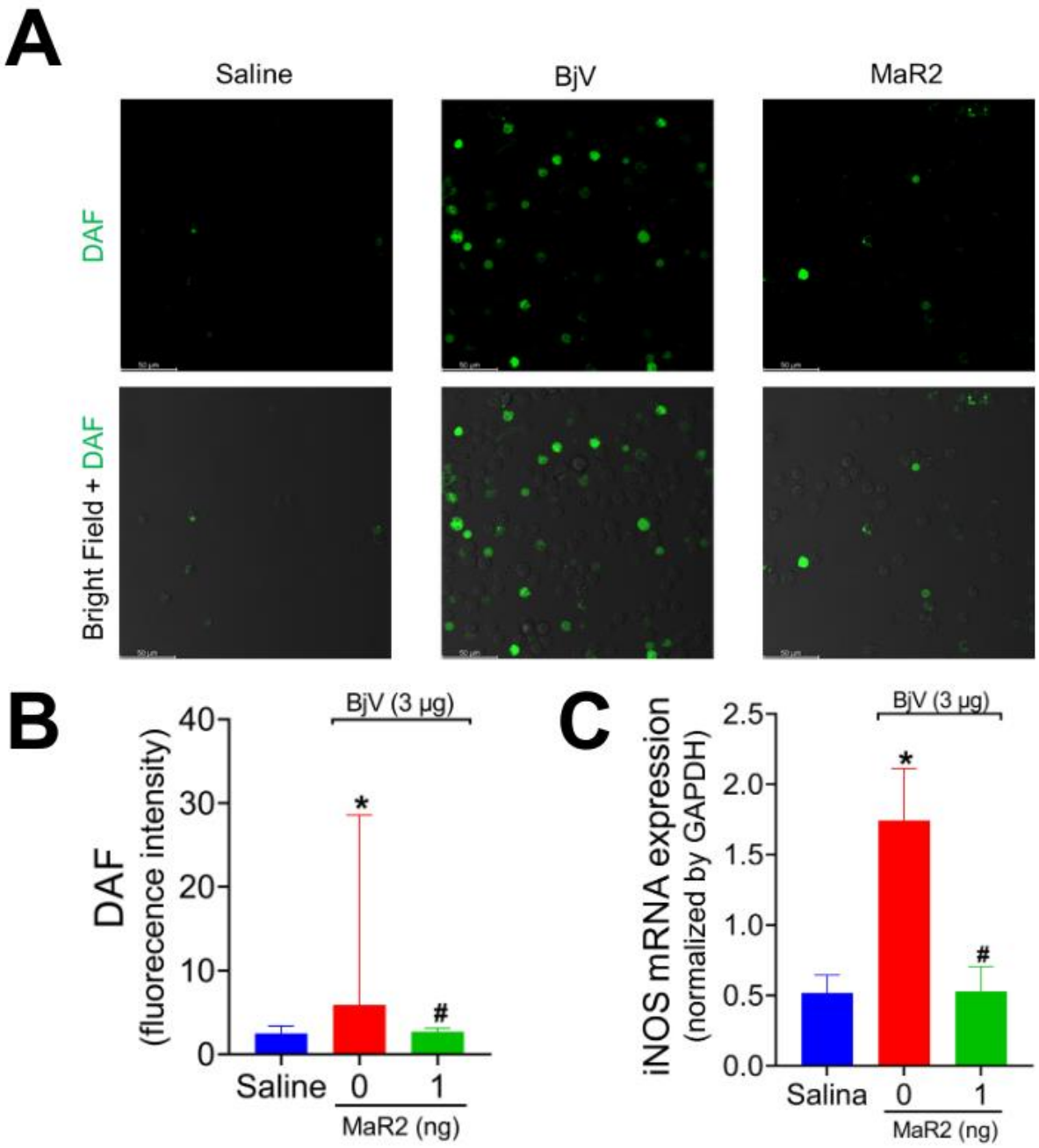

Figure 6. MaR2 reduces nitric oxide levels and iNOS expression on BjV-induced peritonitis. Total NO levels were observed through DAF-FM fluorescent probe (A and B). Results are presented as median with range (B). DAF: n=10 mice per group (*p < 0,05 vs saline; # p < 0,05 vs group 0 [Kruskal-Wallis followed by Dunn's]). iNOS expression was (C) analyzed 7 hours after BjV stimulus using 2−(ΔΔCt) comparative method and Gapdh as constitutive gene. Results are presented as mean ± SEM (C); n = 5 mice per group (* P < 0,05 vs saline; # P < 0,05 vs group 0 [one-way ANOVA followed by Tukey]).

4. Discussion

Snakebite accidents involving snakes represent a serious health problem. In Brazil, 69.3% are associated with the *Bothrops jararaca* snake [2]. *Bothrops jararaca* bites cause pain and inflammation in victims [5], [6]. Many current studies have shown the antioxidant and anti-inflammatory capacity of SPMs, especially maresins [19]–[23]. To date, no one has studied the role of MaR2 in the BjV-induced pain and inflammation model. In the present study, for the first time, the potential role of MaR2 in reducing pain and inflammation was evaluated after BjV-induced inflammatory pain and peritonitis models in mice.

Although the inflammatory and painful state generated after BjV-injection are well-known outcomes, the action mechanisms underlying such effects remain mainly unknown [5], [6]. Spinal cord glial cells, such as microglia and astrocytes, play a central role in modulating hyperalgesia induced by jararhagin, a metalloproteinase found in BjV. To generate this painful state, jararhagin activates the NFκB pathway and induces the production of the pro-hyperalgesic cytokines TNF-α and IL-1β [5]. This occurs due to the expression of cytokine-sensitive receptors at the peripheral ends of nociceptor neurons [37]–[39].

Our results demonstrated that pretreatment with Mar2 (3ng, i.p.) reduced the production of TNF-α, IL-1β and Il-6 in plantar tissue and was also effective in inhibiting inflammatory pain, via reflexive (electronic von Frey methodologies and hot plate) and non-evoked means (static weight-bearing test) to evaluate pain. MaR2 treatment increased mechanical and thermal pain thresholds and allowed for balanced weight distribution between hind paws after BjV injection (1 µg, i.pl.), indicating its efficacy as an analgesic and anti-inflammatory treatment. This effect may be associated with the reduction in TNF-α, IL-1β, and IL-6 levels resulting from MaR2 treatment.

During the acute phase of inflammation, resident cells play a crucial role in releasing pro-inflammatory cytokines and chemokines, facilitating the recruitment of leukocytes to the inflamed site [40]. The recruited cells, mainly polymorphonuclear leukocytes, in particular neutrophils, not only contribute to the production of these cytokines, such as TNF-α, IL-1β and IL-6, but also generate reactive oxygen species (ROS), such as superoxide anion [13]. BjV can cause an abundant leukocyte infiltrate with a predominance of neutrophils in the muscle tissue of mice [41] and intense neutrophil migration in peritonitis, as well as increased levels of IL-6 and IL-1β in the peritoneal cavity [42], [43].

Although neutrophil recruitment is an essential process towards microorganisms' clearance and efficient immune responses [44], it is important to emphasize their role in the production of superoxide anion (O2-) and the release of myeloperoxidases (MPO) - responsible for the generation of cytotoxic oxidants, catalyzing the formation of hypochlorous acid and modulating vascular nitric oxide-dependent signaling cascades [45], [46], as well as in the production of previously mentioned pro-inflammatory and pro-hyperalgesic cytokines [47]–[49].

We observed that pretreatment with MaR2 was able to reduce MPO levels and inhibit superoxide anion production. We verified whether MaR2 would be able to reduce neutrophil recruitment and oxidative stress in plantar tissue and observed that pre-treatment with MaR2 was able to reduce MPO levels and inhibit superoxide

anion production, as well as increase total antioxidant properties (ABTS assay) in the paw tissue stimulated with BjV.

Overall, MaR2 treatment caused a reduction in mechanical and thermal hyperalgesia and an increase in the proportion of weight distribution on the hind paws, which may be related to reduced neutrophil recruitment and levels of pro-inflammatory and pro-inflammatory cytokines hyperalgesic effects TNF-α, IL-1β and IL-6, as well as the reduction of oxidative stress and the increase of antioxidant defenses in the plantar tissue.

In the peritonitis model, BjV can induce the accumulation of leukocytes in the peritoneal cavity, with a predominance of neutrophils in the inflammatory exudate and subsequent increase in concentrations of Thromboxane A2 and Leukotriene B4, contributing to the recruitment and activation of leukocytes at the site of injury induced by BjV [43], mainly mediated by the activity of metalloproteinases and phospholipases A2 present in the venom [10]. Here, we observed that MaR2 (1 ng, i.p.) treatment was able to reduce leukocyte recruitment to the peritoneal cavity, as well as decrease the number of neutrophils recruited to the cavity caused by injection of BjV (3 µg, i.p.).

The production of ROS, the main deleterious agents during the inflammatory process, was assessed, and we observed that pre-treatment inhibited the total intracellular ROS production. This was demonstrated by the reduction in fluorescence intensity of DCF probes (for total intracellular ROS) and DAF probes (for intracellular NO), indicating negative regulation of total intracellular ROS and nitric oxide in inflammatory leukocytes. These results were accompanied by a reduction in O2- production, as observed by spectrophotometry, and the count of cells positive for NBT.

The reduction in reactive oxygen species production may be associated with MaR2's ability to downregulate gp91phox (a subunit of NADPH oxidase responsible for the conversion of molecular oxygen [O2] into superoxide anion [O2-]) [50] and iNOS (an enzyme involved in the significant production of nitric oxide that utilizes l-arginine as a substrate, and molecular oxygen and reduced nicotinamide adenine dinucleotide phosphate (NADPH) as co-substrates) [51], as evidenced by RT-qPCR assays. We observed, then, that MaR2 also can inhibit oxidative stress, acting through the modulation of gp91phox and iNOS mRNA to decrease free radical production in recruited leukocytes.

The presented results reveal that MaR2 treatment demonstrated efficacy in reducing inflammatory pain and processes associated with the acute inflammatory response induced by Bothrops jararaca venom. The data suggest that MaR2 acts comprehensively, diminishing leukocyte migration, reactive oxygen species production, and pro-inflammatory cytokines, while enhancing antioxidant defenses. These findings underscore the therapeutic potential of MaR2 as a promising alternative for the treatment of inflammatory and painful conditions associated with snakebite accidents.

5. Conclusion

In conclusion, our study sheds light on the therapeutic potential of MaR2 in the context of snakebite accidents, particularly those involving the venom of Bothrops jararaca. We demonstrated, for the first time, that MaR2 treatment effectively reduces inflammatory pain and mitigates various aspects of the acute inflammatory response triggered by the venom. This includes a comprehensive impact on leukocyte migration, reactive oxygen species production, and pro-inflammatory cytokines, coupled with an enhancement of antioxidant defenses. The findings underscore MaR2 as a promising alternative for the treatment of inflammatory and painful conditions associated with snakebite accidents, presenting a novel avenue for further exploration in the development of therapeutic interventions in this critical health concern.

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APÊNDICE

APÊNDICE A – Aprovação do comitê de ética animal:

Universidade
Estadual de Londrina

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 103/2021

Londrina, 21 de outubro de 2021.

Prezado(a) professor(a),

Certificamos que o **adendo do projeto** intitulado: **"Avaliação do potencial analgésico dos lipídeos pró-resolução na hiperalgesia induzida pelo veneno de *Bothrops jararaca*"** protocolo CEUA nº **19408.2019.84,** sob a responsabilidade de **Waldiceu Aparecido Verri Junior,** que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina (CEUA/UEL) em **21/10/2021**.

O objetivo deste projeto é avaliar o efeito analgésico e mecanismos dos lipídeos pró-resolução na hiperalgesia induzida pelo veneno de *Bothrops jararaca* (Bjar). **Grau de invasividade: GI3.**

Finalidade	( ) Ensino (X) Pesquisa científica
Vigência da autorização	22/10/2021 a 30/11/2022
Espécie/ linhagem/ raça	Camundongos heterogênicos/ Swiss
Nº de animais	**2.664**
Peso/ Idade	20-25 g/ 30 dias
Sexo	Machos.
Origem	Biotério Central da Universidade Estadual de Londrina/PR.
Amostras a serem coletadas	Tecido plantar, lavado peritoneal, fêmures e tíbias.

Cumpre orientar que caso pretendam-se quaisquer alterações no protocolo experimental aprovado, deve-se submeter o novo protocolo à apreciação da CEUA/UEL anteriormente à execução das modificações.

Em cumprimento às exigências do CONCEA, em até 30 dias da finalização do projeto de pesquisa ou extensão envolvendo o uso de animais (verificar período de vigência expresso neste ofício), é necessário encaminhar relatório da descrição de uso de animais para ceua@uel.br, conforme modelo disponível no site da CEUA: http://www.uel.br/comites/ceua/pages/relatorio-de-projetos.php.

Coloco-me à disposição para quaisquer esclarecimentos que se fizerem necessários. Sem mais para o momento, subscrevo-me, cordialmente,

Profª Drª Maria Fernanda Rodrigues Graciano
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Estadual de Londrina
ceua@uel.br / (43) 3371-5454

Profª Drª Maria Fernanda Rodrigues Graciano
Coordenadora da CEUA/UEL

Ilmo.(a) Sr.(a)
Prof. (a) Dr. (a) Waldiceu Aparecido Verri Junior
Responsável pelo projeto
C/C para a Chefia do Departamento de Ciências Patológicas/CCB
C/C para a Direção do Centro de Ciências Biológicas/CCB
C/C para o Biotério do CCB